FACTORS ASSOCIATED WITH THE CLEARANCE OF UTERINE INFLAMMATION AND RESUMPTION OF OVARIAN CYCLICITY IN POSTPARTUM DAIRY COWS

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FACTORS ASSOCIATED WITH THE CLEARANCE OF UTERINE INFLAMMATION AND RESUMPTION OF OVARIAN CYCLICITY IN POSTPARTUM DAIRY COWS

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Reproductive performance is paramount to efficient milk production in dairy cows. Many postpartum cows do not clear uterine inflammation and resume normal ovarian cyclicity by the time they are presented for reinsemination. This dissertation aimed to study of factors associated with the delay in clearance of uterine inflammation and resumption of ovarian cyclicity in the postpartum cow.

Subclinical endometritis (SCE) is the presence of inflammation in the uterus beyond the normal involution without any other signs of disease. A large epidemiological study was performed obtaining uterine samples, risk factors and reproductive outcome data from 38 commercial dairy herds. Cow-level risk factors for SCE identified were: ketosis, milk production and metritis. Reagent strip test was evaluated as a potential cow-side test for SCE. Reagent strip tests were found to be strongly associated with SCE, however low sensitivity and specificity limits its potential use. The effects uterine sample collections on economically important outcomes on sampled cows were tested and no detrimental effects were found.

The main difference between early postpartum follicles that will ovulate and those that do is the ability to produce a rise in circulating estradiol concentrations. A novel follicle fate prediction method was used to identify cows that will likely go on to ovulate or not using follicle growth parameters and circulating estradiol

concentrations. This allowed for follicular fluid collection and steroid hormone analyses. In non-ovulatory cows; the theca cell function was impaired, there were fewer luteinizing hormone pulses, and had more severe negative energy balance primarily due to decreased feed intake compared with ovulatory cows. Uterine health association with follicular function was evaluated. Certain bacterial isolates were associated with reduced follicle growth. Follicular fluid endotoxin levels were found to be higher in non-ovulatory cow and they also had higher circulating haptoglobin levels which are an indicator for acute phase response.

Negative energy balance and uterine health disorders were associated with both SCE and non-ovulation.

BIOGRAPHICAL SKETCH

Soon Hon Cheong grew up in Seremban, the capital of Negeri Sembilan State in Malaysia, as the third child of four to Yuen Meng Cheong and Kayoko Nakagawa. He was always fascinated with animals growing up and attended veterinary school in University Putra Malaysia. Upon graduation, he came to Cornell University as an Ambulatory and Production Medicine Intern and stayed on for a residency also in Ambulatory and Production Medicine. He then did a second residency at Cornell University this time in Theriogenology in a combined Masters of Science program, becoming a Diplomate of the American College of Theriogenologists after his first year of residency. After his second year in the residency, he received the Graduate Student Assistantship and upgraded his Master of Science to a Doctor of Philosophy program.

This dissertation is dedicated to my lovely wife Lee Yoke Lee and our family.

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LIST OF ABBREVIATIONS

A_0	extrapolated plasma glucose concentration
A_{asym}	asymptotic plasma glucose concentration
AI	artificial insemination
ANOVA	analysis of variance
AUC	area under the curve
BCS	body condition score
BHBA	beta-hydroxybutyrate acid
C.I.	confidence interval
CNCPS	Cornell net carbohydrate and protein system
CV	coefficient of variation
DIM	days in milk
DMI	dry matter intake
DNB	do-not-breed
EB_{post}	postpartum energy balance
EB_{pre}	prepartum energy balance
FIG.	figure
FN	false negative
FP	false positive
FSH	follicle stimulating hormone
GnRH	gonadotropin releasing hormone
GTT	glucose tolerance test
HOMA	homeostatic model assessment
HR	hazard ratio
IGF-1	insulin-like growth factor-1

LE	leukocyte esterase
LH	luteinizing hormone
ME	metabolizable energy
ME30	first test day mature equivalent milk
MP	metabolizable protein
MR	maintenance requirement
NEB	negative energy balance
NEFA	non-esterified fatty acid
NPV	negative predictive value
NRC	National research council
OR	odds ratio
pH	potential hydrogen
PMNL	polymorphonuclear leukocyte
PPV	positive predictive value
QUICKI	quantitative insulin-sensitivity check index
RIA	radio-immuno assay
ROC	receiver operating characteristics
RQUICKI	revised quantitative insulin-sensitivity check index
SCC	somatic cell count
SCC1	first test day somatic cell count
SCE	subclinical endometritis
SD	standard deviation
SE	standard error
Se	sensitivity
SEM	standard error of the mean
Sp	specificity

Spp.	species
T_{50}	half life
TMR	total mixed ration
TN	true negative
TNFα	tumor necrosis factor alpha
TP	true positive
vs	versus

LIST OF SYMBOLS

#	number
%	percent
/	division
<	less than
=	equal
>	greater than
±	plus minus
»	much greater than
≤	less-than or equal to
≥	greater-than or equal to
®	registered
°	degree
C	Celsius
cm	centimeter
d	day
dL	deciliter
e	exponent
Eq	equivalent
et al	and others
EU	endotoxin unit
g	gravitational force
h	hour
k	constant
kg	kilogram

kU	paraoxonase activity unit
lbs	pounds
ln	natural logarithm
log	logarithm
$\log_{10}$	logarithm base 10
Mcal	megacalories
mg	milligram
MHz	megahertz
min	minute
mL	milliliter
mm	millimeter
mMol	millimolar
mo	month
n	number
P	probability
pg	picogram
t	time
™	trade mark
vol	volume
wk	week
x	times
α	alpha
β	beta
ηg	nanogram
μL	microliter
μU	microunit

CHAPTER 1

GENERAL INTRODUCTION

Dairy cow reproductive challenge

Dairy cow reproductive efficiency has been in decline for decades (Lucy, 2001; Thatcher et al., 2006). Successful reproduction is a prerequisite for initiating lactation and milk yield is greatest during the first half of the standard 305-day lactation. At the end of lactation, cows must calve again to initiate a new lactation. Dairy farms that have high reproductive efficiency are able to shorten the interval between calving which decreases the average days postpartum for the cows within the herd putting the average cow closer the peak lactation. Reproductive inefficiency therefore, will increase the interval between calving and reduce the average milk production. In addition, the individual cow that fails to become pregnant in a timely manner will be culled once her milk production drops below the minimal profitable threshold and will have to be replaced which is a considerable cost. Therefore, reproductive efficiency is intimately associated with dairy profitability and reproductive indices are an important gauge of dairy farm success.

Dairy cows are given time to recover from calving and this period, where cows will not be inseminated even when found to be in estrus, is called the voluntary waiting period. In commercial New York dairies, the voluntary waiting period is approximately 60 days after which cows are enrolled in breeding programs. At the end of the voluntary waiting period, approximately 40 to 50% of cows still have uterine inflammation (Gilbert et al., 2005; Hammon et al., 2006; Kasimanickam et al., 2006; Galvao et al., 2009b) while more than 20% of cows submitted for first insemination between 50 and 65 days postpartum are

anovulatory (Cerri et al., 2004; Galvao et al., 2004; Santos et al., 2009). Both the delay of uterine inflammation clearance postpartum and the resumption of ovarian cyclicity are conditions associated with delayed return to reproductive competence postpartum and affected cows have reduced reproductive performance. Increasing our understanding of the conditions will allow further improvement of reproductive management of dairy cows.

Uterine inflammation

All cows develop inflammation of the uterus postpartum and this is part of the normal process of uterine involution. At parturition, virtually all cows have some bacterial contamination and the inflammation aids in clearing the infection (Williams et al., 2005; Sheldon et al., 2008). In addition to fighting infections, the inflammatory process is also involved in histological tissue remodeling of the uterus. At the completion of uterine involution, the uterine inflammation subsides. At 2 weeks postpartum, 100% of cows have uterine inflammation and the proportion of cows with uterine inflammation decreases to 89%, 58%, and 48% at 4, 6 and 8 weeks postpartum (Gilbert et al., 2005). Uterine inflammation that persists is the most common uterine health disorder in dairy cows and this condition is called subclinical endometritis. The variability of disease definition of uterine health disorders including subclinical endometritis in published literature lead to difficulty in interpretation of the findings. In an attempt to standardize uterine health disorder definition (Sheldon et al., 2006), subclinical endometritis is defined as the presence of greater than 18% neutrophils in uterine samples collected between 21 and 33 days postpartum; or greater than 10% neutrophils in uterine samples collected between 34 and 47 days postpartum (Kasimanickam et al., 2004) in the absence of systemic clinical signs. Various

other cutoff points have been proposed based on the optimal cutoff point that is associated with impaired reproductive performance (Gilbert et al., 2005; Barlund et al., 2008; Galvao et al., 2009a) and the difference may be due to the sampling timing, method, and enumeration of cells. Studies comparing diagnostic methods determined cytological evaluation of uterine samples to be superior to ultrasound examination of the uterus and cervix size for diagnosis of subclinical endometritis (Kasimanickam et al., 2004; Barlund et al., 2008). The timing of uterine sample collection is also important and previous work in our lab has shown that the presence of inflammation in uterine samples collected at 21 days postpartum had poor correlation with future reproductive performance while samples collected at 35 and 49 days postpartum were associated with future reproductive performance (Galvao et al., 2009a). Therefore, collection of uterine sample for cytology shortly before the end of the voluntary waiting period for cytological evaluation would be the best method to identify affected cows.

In a previous study, the prevalence of subclinical endometritis within a farm showed large variability ranging from 37% to 74% of cows being affected (Gilbert et al., 2005). The variability indicates a potential to discover factors associated with high and low within-herd prevalence which could be targets for improvement to reduce disease occurrence. There have not been large epidemiological studies of the risk factors for subclinical endometritis in individual cows or farms. Identification of risk factors will give clues to the underlying causes and potentially interventions that can reduce the impact of the disease in affected cows and improve reproductive performance and productivity.

A major challenge in the management of subclinical endometritis is the difficulty in diagnosing affected individuals. Uterine sample collection is time consuming and requires some expertise but another important problem is the time

consuming step of cell enumeration that is required to diagnose cows with subclinical endometritis. A candidate cow-side test is the use of a commercially available leukocyte esterase reagent strip that was originally designed for urinalysis. Leukocyte esterase is present in neutrophils and the use of reagent strips particularly the leukocyte esterase reagent strip is established as an acceptable screening test in urine. Increasing accessibility of subclinical endometritis diagnosis would promote further studies and surveillance of this disease.

Uterine sample collection can be performed by low-volume uterine lavage or by the cytobrush method. The low-volume uterine lavage method is performed by infusing 20 mL of sterile saline solution into the uterus and recovering the infused fluid after mixing with uterine content, while the cytobrush method uses a specially designed pipette with a guarded sheath introduced through the cervix then the brush is extruded and scrapes the uterine wall before reinserting into the protective sheath and removed. The assumption is that the sampling procedure is benign and the cytobrush method has been shown to have no effects on fertility when performed 4 hours after artificial insemination (Kaufmann et al., 2008). However, the effects of sampling have not been tested for low-volume uterine lavage on reproductive outcomes. This must be determined before recommendations can be made to the dairy industry to regularly sample cows for subclinical endometritis diagnosis.

Resumption of ovarian cyclicity

Follicular waves in cattle continue through the first two trimesters of pregnancy until the final month in gestation when follicular waves are impaired by progestagens and the rising estradiol produced by the placenta suppress follicle

stimulating hormone (FSH) pulses (Ginther et al., 1996; Crowe et al., 1998). A rise in FSH occurs in between 1 and 5 days postpartum (Beam and Butler, 1997) and a follicular wave occurs by the second week postpartum. By 10 days postpartum, a dominant follicle can be found in most cows and an increasing circulating estradiol concentration, should result in a surge of luteinizing hormone (LH) and ovulation occurs in 40% of the first-dominant follicle postpartum in dairy cows (Beam and Butler, 1997; Sakaguchi, 2011). Time to first ovulation is highly correlated with the nadir of negative energy balance (NEB) (Canfield and Butler, 1990). The consequence of early resumption of ovarian cyclicity postpartum is an improvement in future reproductive performance (Savio et al., 1990; McDougall et al., 1995; Galvao et al., 2009). Cows that are cycling by 21 days postpartum have better conception rate to the first insemination postpartum than cows that are cycling by 49 days postpartum and cows that are cycling by 49 days postpartum have higher first-service conception rate than cows that are not cycling at 49 days postpartum (Galvao et al., 2009). In a review of anovulatory conditions of postpartum cows (Wiltbank et al., 2002), anovulatory cows can be divided into categories based on maximal follicle diameter namely: only to emergence (< 8 mm), to deviation but not to ovulatory size, and to deviation and ovulatory or greater size including cystic ovarian disease. The underlying pathogenesis is different for each classification of anovulatory condition and is likely to be an effect of severity as only severely sick or under conditioned cows do not develop follicles to deviation and most relatively healthy anovulatory cows develop ovulatory sized follicle but for some reason fail to ovulate (Sakaguchi, 2011). This dissertation will focus only the most common anovulatory condition.

Most cows will develop a dominant follicle in the first follicular wave. The difference between follicles that will go on to ovulate and those that do not is

the extent of the rise in circulating estradiol concentrations initiated by those follicles (Beam and Butler, 1997). Non-ovulatory follicles appear normal by ultrasound examination, however; most do not produce estradiol that is detectable in circulation. Those non-ovulatory follicles that do show a rise in circulating estradiol mostly develop into cystic follicles and the concentrations of estradiol detected are higher than physiological ovulatory estradiol levels. In ovulatory cows, the high circulating estradiol concentrations is detected by the hypothalamus that releases a surge of gonadotropin releasing hormone (GnRH) which in turn stimulates the pituitary to release the LH surge (Crowe, 2008). In non-ovulatory cows that do not develop a rise in circulating estradiol, the potential reason is the impairment of estradiol biosynthesis. Estradiol production by the follicle begins with the uptake of cholesterol by the theca cells which convert the cholesterol to pregnenolone and then to androgens under the influence of LH. The granulosa cells then aromatize the androgens into estrogen under the influence of FSH (Fortune, 1986). This is the two-cell two-gonadotropin model of steroidogenesis (Fortune and Quirk, 1988). Assuming biosynthesis issue, therefore, it is possible then to determine the level of the steroidogenic defect by measuring the intermediate products of steroidogenesis in follicular fluid in steroidogenically defective follicles.

The metabolic state of the postpartum dairy cows is strongly associated with early return to ovarian cyclicity (Beam and Butler, 1997; Opsomer et al., 2000; Wathes et al., 2007). Dairy cows enter a state of NEB during the early postpartum period which reduces the pulsatility of LH (Canfield and Butler, 1990) and increases the negative feedback of the hypothalamus by estradiol (Beam and Butler, 1997). Cows that do not ovulate the first dominant follicle postpartum have decreased dry matter intake by 3 weeks prepartum (Butler et al., 2004). High

energy demands from pregnancy and lactation combined with reduced energy intake result in a rapid mobilization of energy from lipid stores. This increases the concentrations of β-hydroxybutyrate acid (BHBA) and non-esterified fatty acids (NEFA) in cows with NEB which has been associated with impaired follicle functions (Jorritsma et al., 2004; Vanholder et al., 2005). In addition, circulating insulin and insulin-like growth factor-1 (IGF-1) levels are lower in non-ovulatory cows (Butler et al., 2004). Insulin resistance occurs in late gestation and early lactation in high-producing dairy cows and is associated with impaired ovarian function (Butler et al., 2003; Leury et al., 2003; Webb et al., 2004). Insulin resistance prevents lipolysis (Bell, 1995) which increases BHBA and NEFA concentrations but insulin also directly affects follicle function. Both granulosa and theca cells require insulin to be able to respond to gonadotropin stimulation (Poretsky and Kalin, 1987; Stewart et al., 1995; Silva and Price, 2002). Therefore, characterizing energy balance, metabolic and insulin resistance in conjunction with ovarian function will allow better understanding of the physiological state of the cows that have delayed resumption of cyclicity.

Interaction between ovarian cyclicity and uterine health

The development of uterine health disorders and delayed resumption of ovarian cyclicity share some common risk factors. Decreased dry matter intake, low body condition score and NEB have been shown to be risk factors for both (Beam and Butler, 1997; Butler et al., 2006; Hammon et al., 2006; Huzzey et al., 2007; Patton et al., 2007; Crowe, 2008; Sheldon et al., 2008; Galvao et al., 2009; Galvao et al., 2010). There are local effects of the uterus on ovarian function. The first dominant follicle postpartum emerges from the ovary contralateral to the ovary with the corpus luteum from pregnancy which is usually the side of the

uterus that contained the fetus during the previous pregnancy (Kamimura et al., 1993). This selection is lost if hysterectomy is performed even if the corpus luteum is preserved (Thatcher et al., 1991) but the selection is maintained if the previously gravid uterus was present independent of corpus luteum presence (Sheldon et al., 2002). There is an intimate association between venous circulation from the uterus and the arterial circulation to the ovary in cattle which allows efficient transport of prostaglandin $F_{2\alpha}$ produced by the uterus to induce luteolysis. It is therefore, not surprising that other inflammatory products or endotoxin from bacterial contamination of the involuting uterus to be able to exert an inhibitory effect on follicle growth on the ipsilateral ovary.

Bacterial contamination of the uterus postpartum is associated with uterine disease and is also associated with impaired ovarian function (Opsomer et al., 2000; Sheldon and Dobson, 2004; Williams et al., 2007). Cows with more bacterial contamination postpartum have slower follicular growth for the first dominant follicle postpartum, and these follicles have decreased estradiol production (Sheldon et al., 2002; Williams et al., 2007). If ovulation occurs before clearance of bacterial contamination, the progesterone exposure may impair the immune system exacerbating the infection resulting in pyometra (Olson et al., 1984). Administration of exogenous GnRH to early postpartum cows may also result in pyometra (Etherington et al., 1984). Therefore, ovulation before uterine involution is potentially detrimental to uterine health. Conversely, cows that have uterine health disorders that do ovulate may develop prolonged luteal phase (Opsomer et al., 2000; Ranasinghe et al., 2011). Uterine infection results in a shift from the production of prostaglandin $F_{2\alpha}$ which is the luteolytic hormone, to prostaglandin E_2 in uterine tissue (Herath et al., 2009). Uterine health and resumption of ovarian cyclicity are intertwined and both critical to the timely

return to fertility postpartum.

Questions to be addressed by dissertation research

The general objective of this dissertation was the study of factors associated with the delay in clearance of uterine inflammation and resumption of ovarian cyclicity in the postpartum cow.

The first study aimed to identify risk factors for subclinical endometritis both at a cow- and herd level and to characterize the reproductive consequences of the disease. To answer this question, the largest study to date on subclinical endometritis was performed by sampling cows from 38 herds around the state of New York. Risk factor information collected at the time of sampling and herds were followed up to determine the reproductive outcomes of the sampled cows.

The second objective was to validate the use of a reagent strip test as a cow-side test for subclinical endometritis. Samples from the first study were tested to determine if leukocyte esterase, protein and pH reagent strip test could be used individually or in combination as a rapid diagnostic test for subclinical endometritis and associated with impaired reproductive outcomes.

The third objective was to determine the consequence of sampling on economically important parameters of reproductive performance, culling, and milk production. Sampled cows from the first study were compared with un-sampled herd mates to ensure the procedure was benign while generating important data for reproductive management.

The second study was aimed to determine the steroidogenic defect of cows that do not ovulate the first-dominant follicle postpartum. A follicle fate prediction method was tested to allow follicular fluid samples to be collected prior to ovulation and steroid hormone profiles compared between ovulatory and non-

ovulatory follicles. Intensive characterization of the physiological milieu including energy balance, insulin resistance, and endocrine status of ovulatory and non-ovulatory cows was also performed to identify associated factors.

The final objective was to evaluate uterine health effects on the fate of the first-dominant follicle postpartum. Uterine cytology and bacteriology were evaluated with systemic indicators of infection and inflammation on the effects on follicle fate and follicle growth.

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CHAPTER 2

COW-LEVEL AND HERD-LEVEL RISK FACTORS FOR SUBCLINICAL ENDOMETRITIS IN LACTATING HOLSTEIN COWS

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ABSTRACT

The objectives of this study were to obtain prevalence estimates for subclinical endometritis (SCE), determine cow- and herd-level risk factors, and evaluate the reproductive consequences of SCE. A cross-sectional study was used to determine prevalence and risk factors with cows followed in a prospective study to determine reproductive outcomes. Lactating Holstein cows were sampled between 40 and 60 d in milk using low-volume uterine lavage, and cytology was evaluated to determine SCE status. In total, 779 cows from 38 herds were used in the analysis. The cow-level prevalence of SCE was 25.9%. Within-herd level prevalence ranged from 4.8 to 52.6% (median 26.3%, interquartile range 15.6 to 33.3%). Cow-level risk factors identified were ketosis [odds ratio (OR) 3.83; 95% confidence interval (CI) 1.82–8.07], acute metritis (OR 1.86; 95% CI 1.05–3.30], and the interaction between milk production and parity. Primiparous cows that produced more milk had increased odds of having SCE, whereas multiparous cows that produced more milk had decreased odds of having SCE. Herd-level risk factors identified were housing early postpartum cows on bedded packs (herd-level SCE = 36.1%), which increased herd prevalence of SCE by 16.7% (SE 5.58) compared with early postpartum cows housed in freestalls (herd-level SCE = 19.4%), and straw bedding in the calving pen, which decreased herd prevalence of SCE by 10.7% (SE 3.59) compared with herds that used other bedding material. In this study, primiparous cows with and without SCE had similar reproductive performance; however, multiparous cows with SCE had median days open 44 d longer (159 d; 95% CI 126–186 d) compared with unaffected multiparous cows (115 d; 95% CI 106–132 d).

INTRODUCTION

Subclinical endometritis (**SCE**) is a postpartum uterine disease characterized by uterine inflammation in the absence of clinical signs (Sheldon et al., 2006). Gilbert et al. (2005) found the proportion of cows with uterine inflammation diagnosed by cytology to decrease with time, from 100% at 2 wk postpartum to 89, 58, and 41% at 4, 6, and 8 wk postpartum, respectively. Most studies thus far have concentrated on developing diagnostic methods, establishing disease definitions, and describing consequences (Kasimanickam et al., 2006; Barlund et al., 2008). Risk factors for SCE have not been extensively studied at the cow or herd levels. In 5 herds, the prevalence of SCE varied from 37 to 74% at 40 to 60 DIM (Gilbert et al., 2005). This wide range suggests that herd-level risk factors exist that affect the prevalence of this disease. Herd-level risk factors for SCE have not been studied, possibly because of the obstacles of obtaining large numbers of samples for endometrial cytology and the difficulty in obtaining information from enough herds to reach valid conclusions and have precise estimates. Subclinical endometritis impairs reproductive performance by reducing pregnancy to first insemination and increasing median days open (Kasimanickam et al., 2004; Gilbert et al., 2005; Galvão et al., 2009). The economic losses could be substantial in herds with high prevalence of SCE. Indentifying risk factors for SCE may allow for dairy management interventions to aid in controlling this costly disease. Our objectives were to 1) provide cow and within-herd level estimates of SCE prevalence, 2) determine cow- and herd-level risk factors for SCE, and 3) evaluate the effects of SCE on reproductive performance.

MATERIALS AND METHODS

Study population and sampling

The animal procedures performed for this study were approved by the Cornell University Institutional Animal Care and Use Committee. For identification of risk factors and prevalence, a cross-sectional study design was employed and a prospective cohort study was used to determine the effects of SCE on reproductive performance with the experimental unit cows clustered within herds. A convenience sample of 39 commercial dairy herds in New York State with freestall housing and TMR feeding was used based on the farmers' willingness to participate. Inclusion criteria for herds were >400 milking cows and use of DairyComp 305 record-keeping software (Valley Agricultural Software, Tulare, CA). The minimum herd size was used to ensure that adequate eligible cows (approximately 20 cows at 40 to 60 DIM) would be available at the time of sampling. Cows within these herds were eligible to be enrolled if they were between 40 and 60 DIM and before the end of the voluntary waiting period of the farm. Cows were excluded if they 1) were within 2 d of the end of the voluntary waiting period or 2) appeared sick or had abnormal vaginal discharge. Approximately 20 cows were sampled from each herd so that the within-herd prevalence could be estimated with 90% confidence (Win EpiScope 2.0; Thrusfield et al., 2001) assuming a prevalence of 25% based on experience and data from these herds and approximately 30 animals at risk in our sampling window. To represent normal herd demographics, a conscious effort was made to collect approximately one-third of the samples from first-lactation heifers and two-thirds from multiparous cows. Herdsmen were not informed of which cows were sampled or their SCE status until the end of the follow-up period, which was

6 mo after sampling. To limit differences in inter-herd data collection, all farms received a standardized consent form, survey, and case definitions for diseases of interest. Herd information was obtained through a survey completed by the herdsman or owner at the time of sampling. Herd-level risk factors evaluated were herd size, housing of dry cows, housing of cows within the first 30 DIM, calving pen bedding material, number of pen moves during the dry period, number of pen moves during the first 60 DIM, and average projected 305-d mature-equivalent milk yield. Cow-level risk factors were obtained from the dairy herd records collected on the day of sampling. Risk factor categories evaluated were calving related (calving ease scores, calf sex, twins, and stillbirths), postpartum disease (retained placenta, milk fever, acute metritis, ketosis, displaced abomasum), and milking related (first test-day SCC, 305-d mature-equivalent milk yield). At the time of sampling, cows were body condition scored (Ferguson et al., 1994). Data quality was evaluated for each herd. Herds with few recorded disease occurrences were excluded from the cow-level risk factor analysis. Risk factors with data on <50% of sampled animals were excluded from all analysis. Reproductive information (inseminations, pregnancy diagnosis) and censoring (culling and management decision to not breed) were collected at 4 and 6 mo after sampling.

Presence of SCE was determined by cytological evaluation of low-volume uterine lavage as described previously (Gilbert et al., 2005). Briefly, the perineum of the cow was cleansed and a 25-inch Flex Tip sterile plastic infusion pipette (Exodus Breeders Corp., York, PA) was manipulated through the cervix into the uterus. Sterile saline solution (20 mL) was injected into the uterus and agitated gently per rectum; then a sample of the fluid was aspirated. The recovered fluid was centrifuged using a cytocentrifuge directly onto a glass slide. After drying, the slides were fixed and stained using a rapid Romanowsky-type staining procedure

and examined under 400 × magnification. Cells were identified as PMNL (mostly neutrophils), large mononuclear leukocytes (macrophages), small mononuclear leukocytes (lymphocytes), and uterine epithelial cells. Two hundred cells were counted from each slide, and results expressed as a percentage of total cells (excluding erythrocytes). All the slides were read by the same investigator (SHC). Cows were designated as positive for SCE if >10% PMNL were identified, based on the recommendations of Sheldon et al. (2006).

Data management and statistical analysis

Prevalence.

Cow-level prevalence of SCE, as well as within-herd prevalence, was reported as the percentage of cows with >10% PMNL in uterine cytology. Within herd prevalence was used as the dependent variable for herd-level risk factor analysis.

Cow-level risk factors for SCE.

The response variable for the cow-level risk factors was binomial, with cows classified as having SCE or being normal. Because the sampling of study unit was clustered within herds, herd was included as a random effect in all cow-level analyses. Analysis of putative risk factors was performed using PROC GLIMMIX of SAS, version 9.2 (SAS Institute Inc., Cary, NC). Risk factors examined were signalment, which included parity (primiparous or multiparous), days dry, DIM at sampling, and BCS (≥3.5 or <3.5); calving-related risk factors, which included calving ease score (≥3 or <3), twins (twins or singletons), and stillbirths (stillborn or alive); postpartum diseases, which included acute metritis,

retained placenta, milk fever, ketosis, and displaced abomasum (diseased or nondiseased); and milking-related risk factors, which included log first-test-day SCC (**SCC1**) and 305-d mature equivalent milk yield at 30 DIM (**ME30**). Despite circulation of standard disease definitions to herdsmen, disease definition was heterogeneous between herds and for this study was defined as disease occurrence as recorded by farm personnel. Continuous variables were examined for collinearity, outliers, and distribution, and SCC1 was log-transformed to fulfill the normal distribution assumption. Risk factors and interactions with *P*-values < 0.20 were offered to the final model. The final multivariable model was built using manual backward stepwise variable selection with variables retained if $P < 0.05$.

Herd-level risk factors.

For the herd-level risk factor model, the response variable was the proportion of cows with SCE within each herd. Analysis was conducted using PROC GLM of SAS, version 9.2. Herd-level risk factors examined were herd size, average projected 305-d mature-equivalent milk yield, number of moves during the dry period (1 or 2 moves), number of pen moves during the first 60 DIM (1, 2, or 3 moves), bedding material (straw, sand, sawdust, paper, or combination of any of these) in the calving pen, type of housing for early postpartum cows (freestall or bedded pack), and interactions. Herd size was log-transformed. Calving pen bedding material comprised polytomous nominal categories and their combinations. For the risk factors with $P < 0.20$, new categories were created as either using that particular bedding material or not. The final model was built using manual backward stepwise variable selection with variables retained if $P < 0.05$.

Calving to conception interval.

Kaplan-Meier survival analysis (Kaplan and Meier, 1958) was performed to produce survival curves of SCE effects on calving to conception interval alone and after stratification by potential confounders using PROC LIFETEST of SAS, version 9.2. Effect of SCE on calving to conception interval was also evaluated with a multivariable frailty model using STATA version 10 (Stata Corp., College Station, TX) to account for within-herd clustering. Potential confounders evaluated were parity (primiparous or multiparous), BCS (≥3.5 or <3.5), acute metritis, retained placenta, milk fever, displaced abomasum, ketosis, twins, stillbirth, calving ease score (≥3 or <3), log (SCC1), ME30, DIM at first insemination, and interactions. Covariates were evaluated with SCE forced into the model. Cows were censored when culled or as soon as designated do-not-breed by the herdsman. In addition, cows were censored if not pregnant by 210 DIM. The final model was built using manual backward stepwise variable selection with variables retained if $P < 0.05$.

Pregnancy to first insemination.

The effect of SCE on pregnancy to first insemination was examined using PROC GLIMMIX of SAS, version 9.2. Covariates were examined individually in a model with "herd" included as a random variable and SCE forced in as a fixed effect. Covariates evaluated were parity, BCS, acute metritis, retained placenta, milk fever, displaced abomasum, ketosis, log(SCC1), ME30, calf sex, stillbirth, twins, DIM at first insemination, and interactions. To build the final model, variables with $P < 0.20$ were offered and retained if $P < 0.05$ using manual backward stepwise variable selection.

Evaluation of SCE definition.

Receiver operator characteristic curve analysis was performed using JMP8 (SAS Institute Inc.) with pregnancy by 210 DIM as the classifier and percentage PMNL in uterine cytology as the predictor. Optimal cutoff was evaluated overall and by parity as the value giving the greatest sum of sensitivity and specificity.

RESULTS

Descriptive statistics

In total, 812 cows from 39 herds were sampled. One herd was excluded from all analysis as they had recently switched to DairyComp305 for herd recordkeeping, and the quality of data recording was generally poor. In addition, 15 cows were excluded from all analysis for poor quality cytology slides (either too few cells to evaluate or the morphology of the cells was unsatisfactory). Thus, 779 cows from 38 herds had satisfactory cytology and follow-up data. Average DIM at sampling was 46 (SD 6.6); 295 (37.9%) cows sampled were primiparous and 484 were multiparous.

Herd size ranged from 500 to 3,000 milking cows with a median of 880. Examination of herd records revealed unsatisfactory disease records in 4 herds, and cows from those herds had all disease recorded as missing values and were excluded from all cow-level risk factor analyses. The recorded incidences of diseases in the sampled cows were ketosis 5.0%, acute metritis 10.7%, retained placenta 6.6%, displaced abomasum 3.1%, and milk fever 0.1%. Calving-related risk factors analyzed were twinning (3%), calf sex (49.7% heifers), and stillbirths (6.0%). Milk production information (ME30) was available for all cows and the mean was 10,873 kg (23,921 lbs) (SD = 67.4 kg; 148.2 lbs). First test-day SCC

was not tested in all herds; 212 cows had missing observations. The mean DIM at first insemination was later for primiparous cows (69.8 DIM) than for multiparous cows (68 DIM; $P = 0.05$) but was not different by SCE status (68.7 DIM for SCE and 68.7 for unaffected; $P = 0.95$). Calving pen bedding varied with 4 basic bedding materials and 4 combinations. During the follow-up period, 32 cows were designated "do not breed" and were censored at the day of designation, and 74 cows were culled, of which 9 cows were previously classified as "do not breed." In addition, 142 cows were not pregnant by 210 DIM and were censored.

Prevalence

The overall prevalence of SCE was 202 cows affected (25.9% exact binomial, 95% CI 22.3–28.4%) and 577 unaffected. Within-herd prevalence ranged from 4.8 to 52.6% (median 26.3%, interquartile range 15.6 to 33.3%). The distribution of within-herd prevalence is summarized in **Figure 2.1**. The prevalence of SCE in primiparous cows (20.1%) was similar ($P = 0.67$) to that in multiparous cows (26.4%).

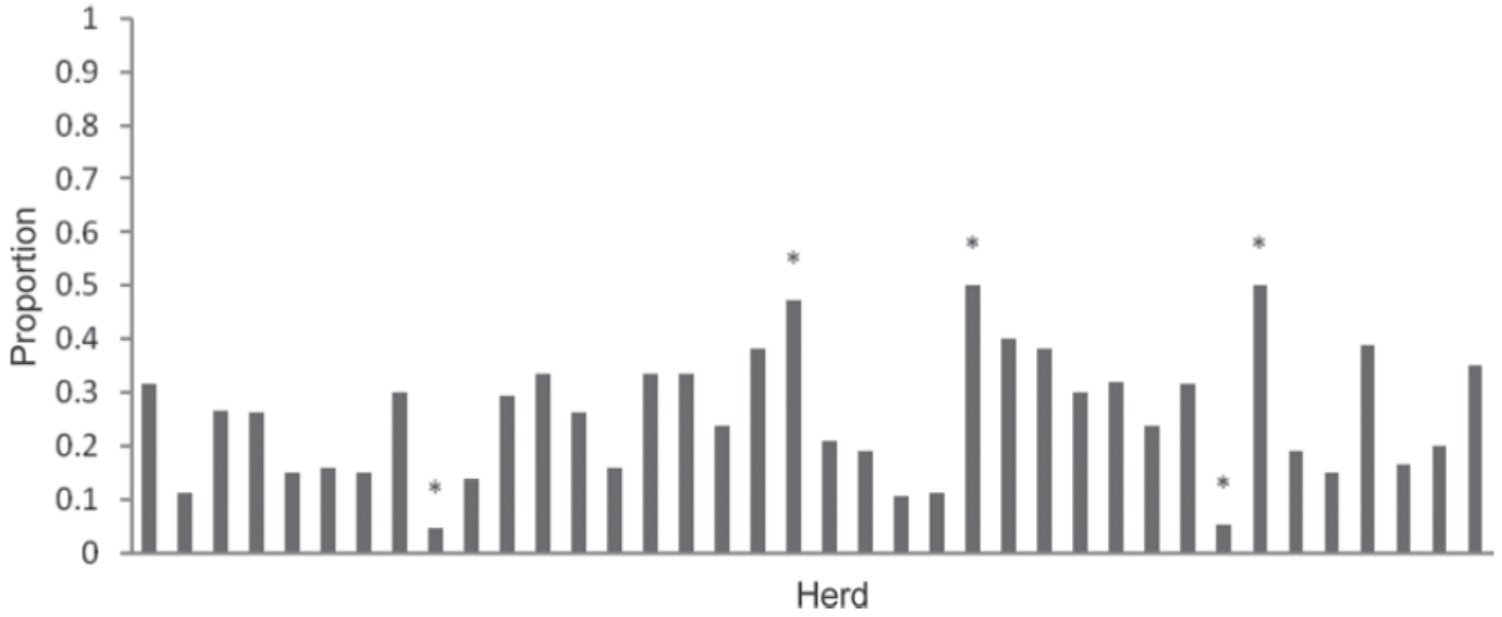

Figure 2. 1

Proportion of cows affected with subclinical endometritis (SCE) by herd arranged in order of sampling. *Herds with SCE prevalence different from the mean, 25.9% ($P < 0.05$).

Cow-level risk factors

Individual risk factor analysis results are summarized in T**able 2.1**. Individual risk factors and interactions with $P < 0.20$ examined in the multivariable analysis were DIM at sampling, acute metritis, retained placenta, ketosis, calf sex, stillbirth, twins, ME30, and the 2 interactions (ME30 × parity and acute metritis × parity). Parity was included to fulfill the hierarchical rule. Manual backward stepwise variable selection resulted in the final model, which included acute metritis, ketosis, and the interaction between ME30 and parity. Parity and ME30 were included to fulfill the hierarchical rule.

To further examine the interaction with parity, the risk factor analysis was repeated stratifying by parity; the results are summarized in **Table 2.2**. Primiparous cows that produced more milk were at increased odds of having SCE, and multiparous cows that produced more milk were at decreased odds of having SCE. In the stratified analysis, metritis was not significant as a risk factor for primiparous cows but primiparous cows with ketosis tended to be at greater odds of having SCE.

Milk yield tended to be significant in primiparous cows, but cows with higher production in this group were at increased risk of SCE compared with multiparous cows. In multiparous cows, ketosis and acute metritis were significant risk factors for SCE, and multiparous cows that produced less milk were at increased risk of SCE.

Table 2.1. Cow-level risk factor estimates for subclinical endometritis (SCE) with herd included as a random effect.

	Individual risk factor			Final model		
Cow-level risk factor[1]	Odds ratio (estimate)	95% CI (SE)	*P*-value	Odds ratio (estimate)	95% CI (SE)	*P*-value
DIM at sampling[2,3]	(0.019)	(0.14)	0.16	—	—	—
Days dry[3]	(−0.0043)	(0.0074)	0.56	—	—	—
Parity (multiparous)	1.10	0.78–1.54	0.60	(4.90)	(1.27)	<0.001
BCS (≥3.5)	0.98	0.68–1.40	0.89	—	—	—
Acute metritis[2]	2.15	1.29–3.60	0.003	1.86	1.05–3.30	0.03
Retained placenta	1.56	0.82–2.97	0.18	—	—	—
Displaced abomasum	1.39	0.55–3.52	0.48	—	—	—
Milk fever	1.80	0.29–11.19	0.53	—	—	—
Ketosis[2]	4.16	2.05–8.48	<0.001	3.83	1.82–8.07	<0.001
Log(SCC1)[3]	(−0.024)	(0.056)	0.67	—	—	—
ME30[2,3]	(0.000049)	(0.000022)	0.03	(−0.0008)	(0.00044)	0.06
Calf sex(bull)[2]	0.80	0.58–1.11	0.18	—	—	—
Stillbirth[2]	1.82	0.98–3.38	0.06	—	—	—
Twins[2]	2.73	1.20–6.18	0.02	—	—	—
ME30 × parity[2,3]	(0.00016)	(0.000049)	<0.001	(0.00019)	(0.000052)	<0.001
Parity × acute metritis[3]	(0.33)	(0.85)	0.08	—	—	—

[1]Log(SCC1) = log-transformed first test-day SCC; ME30 = 305-d mature-equivalent milk yield at 30 DIM.

[2]Risk factors with $P < 0.20$ were offered to build the final model.

[3]Continuous variables reported as regression coefficient and standard deviation.

Table 2.2. Cow-level risk factor estimates for subclinical endometritis stratified by parity with herd included as a random effect.

	Primiparous[1] (n = 231)			Multiparous (n = 424)		
Risk factor	Odds ratio (coefficient)	95% CI (SE)	*P*-value	Odds ratio (coefficient)	95% CI (SE)	*P*-value
Ketosis	2.87	0.85–9.76	0.09	5.62	2.12–14.90	<0.001
Metritis	1.14	0.47–2.78	0.78	2.60	1.19–5.65	0.02
ME30[2,3]	(0.0008)	(0.000045)	0.09	(−0.00011)	(0.000029)	<0.001

[1]No variables were retained after model building for primiparous cows.

[2]ME30 = 305-d mature-equivalent milk yield at 30 DIM.

[3]Continuous variables reported as regression coefficient and standard error.

Herd level risk factors

Herd-level risk factors were examined individually and the univariable analysis is summarized in **Table 2.3**. Calving pen bedding material was identified as a risk factor in the univariable analysis ($P = 0.19$) and was split to 4 dichotomous categories, namely using or not using straw, sand, sawdust, and paper. No significant interactions were found between the main risk factors. Variables offered for building the multivariable model were early postpartum housing, straw bedding in calving pens, and paper bedding in calving pens. In the final model, only early postpartum housing and straw bedding in calving pens was retained. Herds housing early postpartum cows in freestall barns had 16.71% lower herd SCE prevalence (SE = 5.58; $P = 0.005$) compared with herds that housed early postpartum cows in bedded packs. Herds that used straw for calving pen bedding had 10.71% lower herd SCE (SE = 3.59: $P = 0.005$) compared with herds that did not use straw bedding.

Table 2.3. Generalized linear model estimates of herd-level risk factors for subclinical endometritis

	Individual risk factor			Final model		
Herd-level risk factor	Estimate	SE	*P*-value	Estimate	SE	*P*-value
Log(herd size)	0.67	4.92	0.89	—	—	—
Average 305-d mature-equivalent milk yield	0.00067	0.0014	0.63	—	—	—
Early postpartum housing (freestall)[1]	−14.49	6.06	0.02	−16.71	5.58	0.005
Pen moves, dry (1)	−3.56	4.62	0.42	—	—	—
Pen moves, <60 DIM			0.68	—	—	—
(1 vs. 3)	2.55	9.50	0.79	—	—	—
(2 vs. 3)	−1.46	9.71	0.88	—	—	—
Calving pen bedding[1]			0.19	—	—	—
(straw)[1]	−8.87	3.90	0.03	−10.71	3.59	0.005
(sand)	−4.71	6.52	0.47	—	—	—
(sawdust)	1.85	5.21	0.73	—	—	—
(paper)[1]	6.30	4.84	0.19	—	—	—

[1]Risk factors with $P < 0.20$ were offered to build the final model.

Calving to conception interval

Kaplan-Meier survival curves showed that cows with SCE had median days open 26 d longer (142 d, 95% CI 119–159) than that of normal cows (116 d, 95% CI 110–124; $P = 0.02$). Kaplan-Meier survival curves for calving to conception interval were repeated stratifying by SCE status and parity and are shown in **Figure 2.2**. No difference in median days open was observed between primiparous cows with different SCE statuses, but multiparous cows with SCE had 159 d (95% CI 126–186 d) median days open, which was 44 d longer than in multiparous cows without SCE (115 d, 95% CI 106–132 d; $P = 0.006$). A similar interaction was found between parity and BCS on calving to conception interval. Multiparous cows with BCS <3.5 had 41 d longer median days open (148 d) compared with multiparous cows with BCS ≥3.5 (107 d). Body condition score did not affect median days open in primiparous cows (≥3.5 = 118 d, <3.5 = 119 d).

To account for clustering of cows within herds, a frailty model was used to screen covariates for the effect of SCE on calving to conception interval. The interaction between parity and BCS was collinear to the interaction between parity and SCE as described by the Kaplan-Meier survival analysis, and parity × SCE was used to build the final model. The interaction between parity and DIM at first service was not significant ($P = 0.23$) and was dropped from the final model. The final model included parity, BCS, log (SCC1), DIM at first service, and the interaction between parity and SCE. Estimates for the final model and the model stratified by parity are summarized in **Table 2.4.**

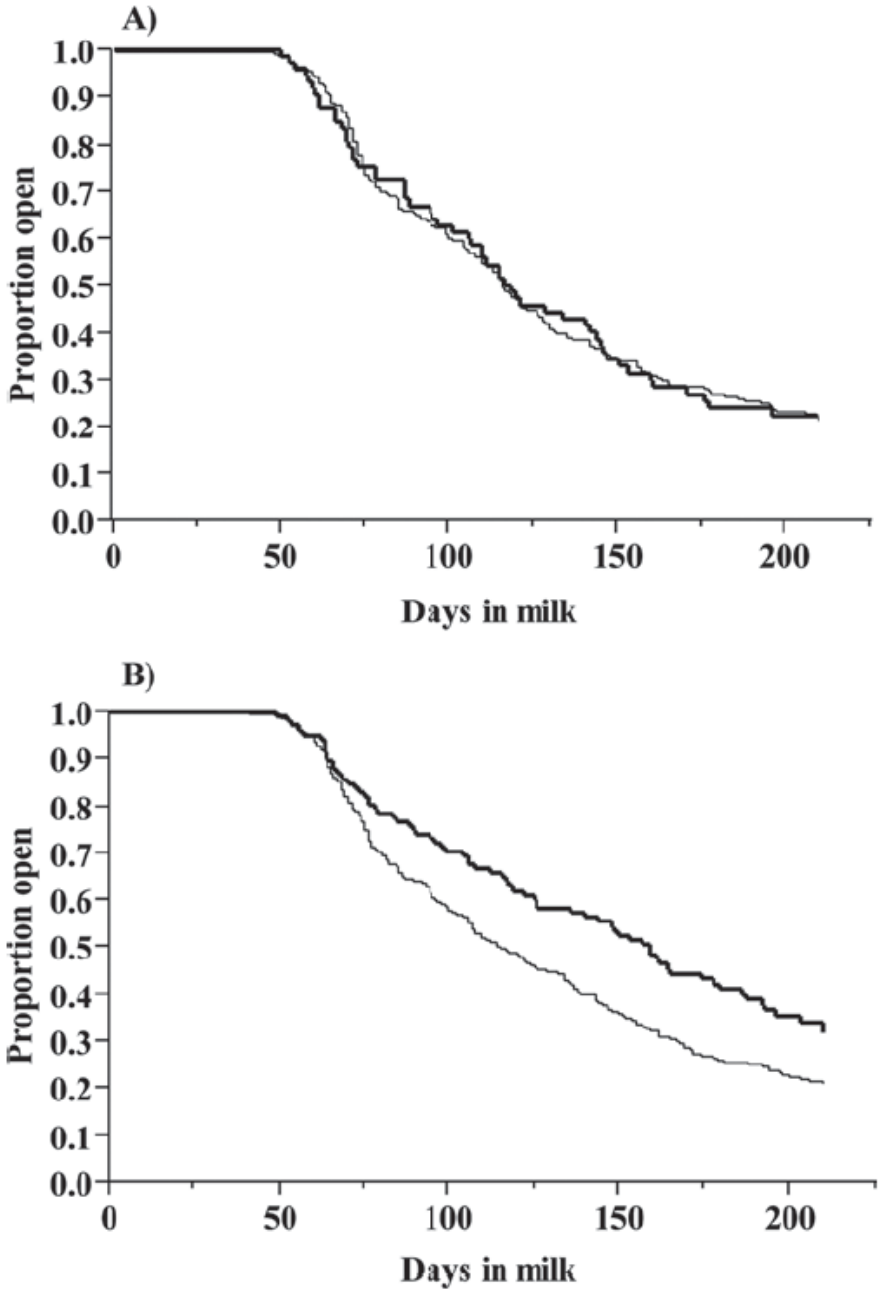

Figure 2. 2

Kaplan-Meier survival analysis for cows with subclinical endometritis (SCE; thick line) compared with normal (thin line) cows: A) SCE did not increase calving to conception interval in primiparous cows ($P = 0.94$); B) multiparous cows with SCE had longer calving to conception interval ($P = 0.005$).

Table 2.4. Final frailty model combined and stratified by parity for the hazard of pregnancy with subclinical endometritis (SCE) forced into the model and herd included as a random effect

	Combined			Primiparous			Multiparous		
Variable	Hazard ratio	95% CI	*P*-value	Hazard ratio	95% CI	*P*-value	Hazard ratio	95% CI	*P*-value
SCE	1.16	0.77–1.76	0.47	1.18	0.76–1.83	0.45	0.68	0.49–0.94	0.021
BCS (≥3.5)	1.29	1.03–1.63	0.03	0.92	0.62–1.35	0.66	1.29	1.03–1.63	0.029
Log(SCC1)[1]	0.74	0.61–0.91	0.004	0.71	0.50–1.01	0.053	0.79	0.61–1.01	0.065
DIM at first service	0.99	0.98–1.00	0.01	0.99	0.9 –1.00	0.13	0.98	0.97–1.00	0.018
Parity[2]	1.11	0.86–1.45	0.42	—	—	—	—	—	—
Parity × SCE[2]	0.58	0.34–0.99	0.05	—	—	—	—	—	—

[1]Log-transformed first test-day SCC.

[2]Parity and the interaction between parity and SCE were dropped in the stratified model due to collinearity.

Pregnancy to first insemination

A summary of individual covariates in a model with herd included as a random effect and SCE forced in as a fixed effect is shown in **Table 2.5**. No significant interactions were found and only log (SCC1) and BCS were evaluated to build the final model, in which only BCS was retained. Cows with SCE had lower odds (odds ratio = 0.61, 95% CI 0.40–0.93; $P = 0.02$) for pregnancy at first insemination compared with cows without SCE. Cows with BCS ≥3.5 had higher odds of pregnancy to first insemination (odds ratio = 1.78, 95% CI 1.24–2.56; $P = 0.002$) compared with cows with BCS <3.5.

PMNL cutoff for SCE definition

The overall sensitivity and specificity for the 10% PMNL cutoff for predicting pregnancy by 210 DIM were 0.79 and 0.43, respectively. The cutoff with the highest combined sensitivity and specificity was 9.5% PMNL, which is very close to the published recommendation of 10% PMNL. When stratified by parity, PMNL was not very predictive of pregnancy by 210 DIM ($P = 0.66$) for primiparous cows, with the optimal cutoff 30% PMNL (sensitivity = 0.93, specificity = 0.13). In multiparous cows, however, the optimal cutoff point was 9.5% PMNL (sensitivity = 0.80, specificity = 0.36) and was predictive of pregnancy by 210 DIM ($P = 0.005$).

Table 2.5. Coefficients for subclinical endometritis (SCE) effect on pregnancy to first insemination, with herd included as a random effect

	Individual factors			Final model		
Variable[1]	Odds ratio (coefficient)	95% CI (SE)	*P*-value	Odds ratio	95% CI	*P*-value
SCE[2]	0.52	0.34–0.81	0.004	0.61	0.40–0.93	0.02
Parity (primiparous)	1.18	0.85–1.64	0.32			
BCS ($\geq$3.5)[2]	1.78	1.24–2.56	0.002	1.78	1.24–2.56	0.002
Acute metritis	0.83	0.47–1.49	0.54	—	—	—
Retained placenta	0.78	0.38–1.60	0.50	—	—	—
Milk fever	1.83	0.29–11.45	0.52	—	—	—
Displaced abomasum	0.76	0.267–2.11	0.59	—	—	—
Ketosis	0.58	0.23–1.46	0.25	—	—	—
Log(SCC1)[2,3]	(0.11)	(0.05)	0.03	—	—	—
ME30[3]	(0.00003)	(0.000021)	0.23	—	—	—
Calf sex (bull)	1.189	0.86–1.64	0.29	—	—	—
Stillborn	1.059	0.54–2.07	0.87	—	—	—
Twins	0.983	0.40–2.42	0.97	—	—	—
DIM at first service[3]	(0.0054)	(0.0073)	0.46	—	—	—

[1]Log(SCC1) = log-transformed first test-day SCC; ME30 = 305-d mature-equivalent milk yield at 30 DIM.

[2]Risk factors with $P < 0.20$ were offered to build the final model.

[3]Continuous variables reported as regression coefficient and standard error.

DISCUSSION

The 779 cows from 38 herds sampled in this study make this the largest observational investigation of SCE in dairy cows to date. Herd-level risk factors were suspected to be substantial based on previous work by Gilbert et al. (2005), which showed large variability in the proportion of cows with SCE between herds, and part of our aim was to identify herd-level risk factors. This required a large number of herds, which, in turn, needed to be accounted for in the analysis.

The most significant cow-level risk factors for SCE were ketosis and milk production interaction with parity. Clinical ketosis is an indicator of severe negative energy balance (Duffield et al., 2009). Negative energy balance has been implicated in reduced reproductive performance primarily through impairment of the gonadotropin and somatotropin axes (Chagas et al., 2007) and more recently in uterine immune function (Hammon et al., 2006; Wathes et al., 2009). Ospina et al. (2010) described a profound effect of even subclinical ketosis on reproductive success. Subclinical endometritis is likely the result of impaired uterine immune function due to negative energy balance and a mechanism of energy balance affecting reproduction. The interaction between milk production and parity was strongly associated with the risk of SCE. We are not able to explain why primiparous and multiparous cows behaved differently with regard to milk yield and disease, but our current results, in which primiparous cows that produced more milk were at higher risk of SCE and multiparous cows that produced more milk had lower risk of SCE, reflect those of Galvão et al. (2010).

The only other significant risk of SCE was acute metritis. The odds ratio of 1.86 found in our study is slightly lower than the >2-fold increased risk reported by Rutigliano et al. (2008). Other postpartum diseases have been reported to increase the

risk of developing SCE. Kasimanickam et al. (2004) reported retained placenta and assisted calving as important risk factors for SCE, and Rutigliano et al. (2008) found a tendency for cows with retained placenta to develop SCE but retained placenta was not significant in this study. Milk fever has previously been reported to be a risk factor for clinical endometritis (Whiteford and Sheldon, 2005). However, we did not find a correlation between clinical milk fever and SCE. This discrepancy could be due to unrecorded prophylactic calcium administration by herd managers, or failing to record clinical disease as discussed earlier. No diagnostic tests were performed; therefore, milk fever, and particularly subclinical hypocalcemia, cannot be ruled out as an important risk factor. The prevalence of SCE has been reported to be similar in primiparous and multiparous cows (Gilbert et al., 2005; Rutigliano et al., 2008; Galvão et al., 2009), and this finding was repeated in the current study. Days postpartum were not significant in our study perhaps because of the relatively small variation in the timing of sampling, with all samples being obtained between 40 and 60 DIM.

Herd-level risk factors have not been described previously. The prepartum period appears to be a critical period for future risk of metritis. Huzzey et al. (2007) found that DMI is reduced in cows 2 wk before metritis; aggressive behavior among cows in the dry period is also reported as a risk factor. Changing groups may lead to aggressive behavior and decreased feed intake, which lead to the inclusion of pen moves in the dry and early postpartum period as a potential risk factor. However, the number of moves may not provide a reflection of the stress suffered by cows because several other management factors involved in the manner of making pen moves play a major role. Because the number of pen moves is decided on a herd basis, it was included as a potential herd-level risk factor. Based on our data, pen moves did not affect herd prevalence of SCE. Bedding material in the calving pens and early

postpartum housing was the only significant risk factor at the herd level. Only 4 herds housed early postpartum cows on bedded packs and those herds had higher herd prevalence of SCE. Because of the small numbers, interpretations should be made with caution. Straw was used as bedding in calving pens in 21 of 38 herds, and those herds had lower herd prevalence for SCE. Although further studies need to be done to determine if changing to straw bedding and freestall housing for early postpartum cows will result in lowering of herd SCE prevalence, these represent potential interventions that were not previously identified.

Subclinical endometritis caused reproductive impairment with a longer calving to conception interval. Perhaps the most surprising finding was the difference between the effects of SCE on hazard of pregnancy between primiparous and multiparous cows, in which primiparous cows were not affected and all the observed difference was in multiparous cows. This finding provides a potential for targeted treatments and prophylaxis. In addition, thin multiparous cows were affected more severely by the negative reproductive effects of SCE (increase of 45 median days open) even though multiparous cows with good body condition were also affected (increase of 25 median days open). This may be a further manifestation of the effects of negative energy balance on uterine health. A large number of cows had missing values for log (SCC1) and BCS, resulting in the low number of observations in the final frailty model. A reduced frailty model was repeated containing only parity, SCE, and parity × SCE using all observations, and found the interaction parity × SCE to have a hazard ratio for pregnancy of 0.68 (95% CI 0.45–1.02; $P = 0.059$). Log (SCC1) was identified as the most significant covariate that increased calving to conception interval ($P = 0.002$). High SCC has been reported to decrease reproductive performance (Pinedo et al., 2009).

First-service conception rate was found to be significantly lower for cows with

SCE, in agreement with previous reports (Gilbert et al., 2005; Barlund et al., 2008). The effects of SCE were also evaluated for subsequent inseminations, and the associations were similar but smaller than for the first insemination (data not shown). Surprisingly, parity was not significant in this model even though the first-service conception rate difference for primiparous cows with SCE (28.4%) and unaffected primiparous cows (30.3%) was smaller, numerically, than the difference between multiparous cows with SCE (18.0%) and unaffected multiparous cows (29.5%). First test-day SCC was not retained in the final model for first-service conception, even though it had a greater effect than SCE on calving to conception interval. First test-day SCC and its interactions were not risk factors for SCE. These results suggest that the causes of inflammation in the udder and uterus are different and, although both affect reproductive performance, SCE appears to have greater effects on the first insemination.

The possibility of information bias arose from the nonuniformity of risk factor diagnosis and recording. Milk production and reproductive information are usually reliable and uniform across different herds, but disease occurrence and calving-related information are less reliable and occasionally not recorded at all. We attempted to reduce this bias first by examining the herd records for unusual patterns of each risk factor occurrence in each herd. Four herds had unusually low disease occurrence and were excluded for all cow-level risk factor analyses. The exclusion of these 4 herds did not change the odds ratio estimates or the final cow-level risk factor model although the *P*-values were decreased slightly. Several herds did not have good records of certain disease occurrence, particularly milk fever and, to a lesser extent, retained placenta. The poor recordkeeping for milk fever and retained placenta may have contributed to the inability to detect any association between these diseases and our outcomes of interest. In addition, some calving-related risk factors, particularly

calving ease score, were poorly recorded and were excluded. In addition to screening the data, inclusion of herd as a random variable helps account for some herd variability.

Diagnosis of SCE depends on the proportion of PMNL in uterine cytology, estimation of which is repeatable between evaluators with kappa values from 0.76 to 0.85 (Gilbert et al., 2005; Barlund et al., 2008). To prevent further variability, all the slides were read by the same investigator and most samples (>90%) were collected by the same investigator. The herds sampled were all located within New York State but the average travel time was about 3 h from the herd to the college, with the furthest herd located over 6 h away. Some samples were unacceptably degraded when they were not processed promptly, usually when 2 herds were sampled on the same day. The presence of mucus in some of the samples resulted in poor adhesion of cells to the slides, resulting in some slides not having sufficient cells to make a definitive diagnosis. Only one slide was produced per sample and making duplicates could have rescued some samples. However, only 15 samples were lost in this manner, which is < 2% of the samples collected. Kasimanickam et al. (2004) reported difficulty in retrieving fluid from the low-volume uterine lavage but that was not experienced in this study. The optimal cutoff point for PMNL to define SCE using receiver operator characteristic curve analysis with pregnancy at 210 DIM agreed with the recommendations of Sheldon et al. (2006).

CONCLUSION

Cow-level risk factors identified for SCE were ketosis, acute metritis, and interaction between parity and milk production, in which primiparous cows with higher milk production were at higher risk and multiparous cows with higher production were at lower risk for SCE. Herd-level risk factors were early postpartum housing in bedded packs and calving pen bedding not using straw, although only 4 herds used bedded pack and thus this finding should be interpreted carefully. In our study, multiparous cows with SCE had increased calving to conception interval but primiparous cows with SCE were not observed to have the same impairment.

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CHAPTER 3

USE OF REAGENT TEST STRIPS FOR DIAGNOSIS OF ENDOMETRITIS IN DAIRY COWS

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ABSTRACT

The use of leukocyte esterase (LE), protein and pH tests were evaluated on widely available urinary test strips (Multistix® 10SG) on uterine lavage samples as a potential cow-side test for the diagnosis of cytological endometritis. Uterine lavage samples of 563 lactating Holstein cows between 40 and 60 d postpartum from 28 herds were evaluated. Endometrial cytology was used as the reference for endometritis, with a cutoff point of ≥ 10% neutrophils. All three LE, protein and pH were increased in cows with cytological endometritis and the associations were highly significant. Optimal cutoff points determined by receiver operating characteristic analysis for LE, protein and pH were ≥ ++, ≥ 300 mg/dL, and ≥ 7.0, respectively. Combining the results for LE and pH improved the performance of the test strip but this resulted in a group of cows (20.6% of cows) which were approximately equally likely (46 % with endometritis and 54% without endometritis) to have cytological endometritis or not and therefore cannot be accurately classified. The direct relationship between reagent strip test and reproductive performance was also evaluated. Reproductive impairment due to endometritis was restricted to multiparous cows and significantly decreased reproductive performance was observed for multiparous cows with lavage fluid LE ≥ +++ (154 vs 115 median days not-pregnant) as well as cows with pH ≥ 7.0 (150.5 vs 111.5 median days not-pregnant) but not in cows with high protein, even at the highest cutoff point. In conclusion, reagent strip test results were strongly associated with cytological endometritis and reproductive impairment; however, in comparison to conventional cytology, the performance of reagent strip as an alternative test was relatively poor and may require further refinement.

INTRODUCTION

Endometritis is an inflammatory uterine disease that persists beyond normal uterine involution and impairs reproductive performance (Barlund et al., 2008; Gilbert et al., 2005; Kasimanickam et al., 2004; Cheong et al., 2011). Affected cows frequently show no external symptoms (Sheldon et al., 2006; Dubuc et al., 2010). Diagnostic methods such as ultrasonographic evaluation of the reproductive tract and uterine content are inferior to cytological examination of uterine content (Barlund et al., 2008; Kasimanickam et al., 2004) which lead to the proposed disease definition based on cytology as the presence of > 18% neutrophils in uterine samples collected between 21 and 33 d post partum or > 10% neutrophils between 34 and 47 d in the absence of purulent vaginal discharge (Sheldon et al., 2006). Cytologic evaluation of uterine samples is currently the best method to diagnose inflammatory disease of the uterus. In a farm setting, however, this method is inconvenient as it involves collection of the sample, preparation of the slides and staining followed by microscopic examination and identification and enumeration of cells. Uterine samples collected using the cytobrush method (Barlund et al., 2008; Kasimanickam et al., 2005) allow easier slide preparation compared with samples collected using low-volume uterine lavage but still require the time-consuming cell evaluation step. The lack of a practical cow-side test is a major reason endometritis is not monitored or managed in commercial herds.

A candidate cow-side test for the diagnosis of endometritis from uterine lavage fluid is leukocyte esterase (**LE**), for example on a reagent strip intended for urinalysis, such as Multistix® 10 SG (Bayer Corporation, Elkart, IN, USA). In a smaller study, Santos et al. (2006) reported high sensitivity (83%) and specificity (94%) when using LE strip to diagnose endometritis. Multistix® 10 SG is a reagent strip of 10 tests, namely: LE, nitrite, urobilinogen, protein, pH, blood, specific gravity, ketone (acetic

acid), bilirubin and glucose. The LE compound is present in neutrophils and therefore, a positive result of this test is the most direct indicator of inflammatory cells in urine using reagent strips. In addition, protein and pH reagent tests may be useful in the diagnosis of endometritis as well as providing insight into the pathogenesis of the condition. Fluid accumulation in the uterine lumen is used as an indicator of inflammation (Barlund et al., 2008; Kasimanickam et al., 2004) which, if present, could elevate the protein content of the recovered fluid of low volume uterine lavage making protein concentration a potential diagnostic test. Inflammation of the udder or vesicular glands elevates the pH of milk (Marshke and Kitchen, 1985) and seminal fluid (Juneja et al., 1965), respectively, but it is unknown if inflammation of the uterus is associated with an elevation of pH in uterine fluid.

The objectives of this study were: 1) to determine if LE, or protein, or pH, or a combination of reagent strip tests were associated with cytological endometritis, 2) to identify cut-off points for associated reagent tests based on cytology and reproductive outcome and 3) to identify other factors associated with LE, protein and pH in uterine lavage samples.

MATERIALS AND METHODS

Sample collection

Uterine lavage samples used in this experiment were part of a larger study (Cheong et al. 2011). The present study was initiated after 10 herds had already been sampled. All samples collected from that point on were included in the present study. Selection of herds for the study was from a convenience sample of herds that were willing to participate in the study. The inclusion criteria for herds sampled were: located in New York State, large herd size (minimum of 400 milking cows) and used DairyComp 305 (Valley Ag Software, Tulare, CA, USA) for keeping herd records. The inclusion criteria for cows sampled were: between 40 and 60 d postpartum, apparently healthy (by cursory visual examination), no external vaginal discharge observed by visual examination, not inseminated and at least 2 d before the end of the voluntary waiting period for that specific farm (average 59 d; range 50 - 70). Herd records were obtained at the time of sampling and reproductive outcomes were obtained by follow-up herd records collected at 4 and 6 months after sampling.

Animal procedures were approved by the Cornell University Institutional Animal Care and Use Committee. Uterine lavage samples were obtained as previously described (Gilbert et al., 2005). Briefly, paper towels were used to cleanse the perineum of the cow then a 63.5 cm sterile flex tip infusion pipette (Exodus Breeders Corporation, York, PA, USA) was introduced into the uterus through the cervix. Twenty mL of sterile saline solution (0.9% Sodium Chloride Injection USP; Baxter Healthcare Corp, Deerfield, IL, USA) was then infused into the uterus. Approximately 5- 8 mL of fluid was recovered by aspiration. The samples were put on ice and transported to the laboratory for analysis. One drop of uterine lavage sample was added to each test on the Multistix® 10 SG reagent strip. Protein and pH results were read after 1 min and the LE result read after 2 min as per manufacturer

instructions. Protein results were recorded in 6 categories which were: negative, trace, + (30 mg/dL), ++ (100 mg/dL), +++ (300 mg/dL) and ++++ (>2,000 mg/dL); pH results were recorded in 7 categories: 5.0, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5; and LE results were recorded in 5 categories: negative, trace, + (small), ++ (moderate) and +++ (large). Cytological evaluation of the uterine lavage samples was performed after cytocentrifugation (105 g for 3 min) and staining using Camco® stain pak stain (Cambridge Diagnostic Products Inc. Fort Lauderdale, FL, USA) by counting 200 cells (neutrophils, lymphocytes, macrophages and uterine epithelial cells excluding erythrocytes) and results were expressed as the percentage of total cells. Cows were considered to be positive for endometritis if neutrophils were >10% of total cells (Sheldon et al., 2005).

Data management and statistical analysis

Association of reagent strip results with cytologic endometritis.

All three LE, protein, and pH results were recorded as ordered categories. The categories of LE 'Negative' and protein 'Negative' had <5 observations that were positive for endometritis and these categories were combined with 'trace' for all analyses. To test the hypothesis that the reagent strip results were associated with endometritis, a multivariable logistic regression model was produced using PROC GLIMMIX of SAS version 9.2 (SAS Institute, Cary, NC, USA) with cytological endometritis as the dependent variable. Herd was included as a random effect. The association between LE, protein and pH were tested individually with cytological endometritis and a final model was built for each reagent strip test. In addition to reagent strip test results, fixed effects considered were: parity (primiparous or multiparous), body condition score (≥ 3.5 or < 3.5), ketosis, metritis, retained placenta, (disease or no disease), Log (first test-day somatic cell count), days postpartum at

sampling and two-way interactions. Disease occurrence data for ketosis, metritis and retained placenta were according to herd records with the diagnosis made by the herdsman. The final model was built using manual backwards stepwise variable selection and variables were retained if $P < 0.05$.

To evaluate reagent strip as a diagnostic test for endometritis, the reagent strip test results were dichotomized (positive test or negative test) at all possible cutoff levels. A series of 2 x 2 tables was created for reagent strip test against cytologic evaluation. True positive, true negative, false positive and false negative results were recorded and sensitivity (**Se**), specificity (**Sp**), positive predictive value (**PPV**) and negative predictive value (**NPV**) were calculated using those values at the apparent prevalence in these herds. The cutoff point with the highest sum of sensitivity and specificity was selected as optimal. Receiver operating characteristic (**ROC**) analysis was performed using MedCalc version 11.5 (MedCalc Software, Mariakerke, Belgium) and the area under the curve and *P*-values are reported.

Association of reagent strip results with reproduction.

Reproductive outcome was examined by Cox proportion hazards model using days from calving to subsequent pregnancy as the event of interest. Incomplete observations were right censored when the cows were culled, designated "Do-Not-Breed" by the herdsman or at 210 days-in-milk. Reagent strip results were dichotomized at all levels and were tested individually and sequentially from the lowest threshold to the highest for effect on calving-to-conception interval using Stata version 10 (Stata Corp, College Station, TX, USA) including herd as a random (shared frailty) effect. The model controlled for the effects of parity (primiparous or multiparous), body condition score (≥ 3.5 or < 3.5), ketosis, metritis, retained placenta, displaced abomasum (disease or no disease), Log (first test day somatic cell count),

days postpartum at first-insemination and two-way interactions. Models were built by manual backward step-wise exclusion of variables. If the interaction with parity was $P < 0.05$, the analysis was stratified by parity. Variables were retained in the final model if $P < 0.05$.

Factors associated with reagent strip tests.

The purpose of this analysis was to determine if there were factors that affected LE, protein and pH results other than cytological endometritis. A multivariable logistic regression model was produced with PROC GLIMMIX of SAS version 9.2 with cumulative logit function and reagent strip results as the dependent variable. The variables tested methods for model building were the same as described in section 2.2.1 except endometritis was tested as a fixed effect in addition to the described list of variables.

Determination of combination cutoff-points.

Reagent strip possible results were dichotomized to all possible combination and evaluated for Se, Sp, PPV and NPV to detect cytological endometritis. The optimal combination was evaluated for first-service conception rate and calving-to-conception interval using the PROC GLIMMIX of SAS version 9.2 and Stata version 10, respectively, as described for individual reagent strip test.

RESULTS

Descriptive statistics

In total, 563 cows from 28 herds were included in the study. The median herd size was 895 (range = 540 – 3000) milking cows and the average projected 305 d mature-equivalent milk production was 12,562 (S.D. = 620) kg of milk. Overall prevalence of cytological endometritis was 27.7 % (156/563), while the average within-herd prevalence was 27.8 % (range = 5.3 % to 52.6 %).

Reagent strip compared to cytology

All three LE, protein and pH were strongly associated with cytological endometritis and retained in the respective final models. Ketosis was retained in all three final models, and metritis was retained in the final model for protein reagent test association was cytological endometritis. The proportions of cows with endometritis increased as LE, protein and pH values increased. Herd was not significant as a random variable for any of the reagent strip tests models ($P > 0.15$).

Dichotomized reagent strip results categorized by endometritis disease status determined using lavage are summarized in **Table 3.1**. Receiver Operator Characteristics analysis showed the optimal cut-off points to be ≥ ++ for LE (area under the ROC curve (AUC) = 0.69; $P < 0.0001$), ≥ +++ for protein (AUC = 0.60; $P < 0.001$) and ≥ 7.0 for pH (AUC = 0.64; $P < 0.001$) to be used for the diagnosis of endometritis. At the optimal cutoff point, the LE test had Se = 76.9%, Sp = 51.8%, PPV = 38% and NPV = 85.4%. At the optimal cutoff point the protein test had Se = 58.3%, Sp = 55.8%, PPV = 33.5% and NPV = 77.7%. At the optimal cutoff point, the pH test had Se = 44.9%, Sp = 78.4%, PPV = 44.3 and NPV = 78.8.

Table 3.1. Performance of Multistix 10 SG (Bayer Corporation) LE, protein, and pH reagent strip for diagnosis of endometritis from uterine lavage samples collected from postpartum dairy cattle.

Test	Cutoff	TP (N)	TN (N)	FP (N)	FN (N)	Se (%)	Sp (%)	PPV (%)	NPV (%)
LE	≥ Trace	152	28	379	4	97.4	6.9	28.6	87.5
	≥+	145	74	333	11	92.9	18.2	30.3	87.1
	≥++	120	211	196	36	76.9	51.8	38.0	85.4
	≥+++	53	367	40	103	34.0	90.2	57.0	78.1
Protein	≥+	147	47	360	9	94.2	11.5	29.0	83.9
	≥++	133	98	309	23	85.3	24.1	30.1	81.0
	≥+++	91	227	180	65	58.3	55.8	33.6	77.7
	≥++++	31	367	40	125	19.9	90.2	43.7	74.6
pH	≥6.5	101	219	188	55	64.7	53.8	34.9	79.9
	≥7.0	70	319	88	86	44.9	78.4	44.3	78.8
	≥7.5	49	349	58	107	31.4	85.7	45.8	76.5
	≥8.0	30	373	34	126	19.2	91.6	46.9	74.7
	≥8.5	18	392	15	138	11.5	96.3	54.5	74.0

Protein results were recorded in six categories which were: negative, trace, + (30 mg/dL), ++ (100 mg/dL), +++ (300 mg/dL), and ++++ (>2000 mg/dL); and LE results were recorded in five categories: negative, trace, + (small), ++ (moderate), and +++ (large).
FN, false negative; FP, false positive; LE, leukocyte esterase; NPV, negative predictive value; PPV, positive predictive value; Se, sensitivity; Sp, specificity; TN, true negative; TP, true positive.

Reagent strip and reproduction

Elevated LE was significantly associated with decreased hazard of pregnancy (increased calving-to-conception interval) only at the highest cutoff point of ≥ +++ (144 median days not-pregnant for LE ≥ +++ and 117 median days not-pregnant for LE < +++ cows; HR = 0.76, 95 % C.I. 0.57 – 1.00; *P* = 0.05). The interaction of parity and LE was significant (*P* = 0.04), therefore, further analysis were stratified by parity. Stratification by parity showed the effects to be limited to multiparous cows where LE ≥ +++ (154 median days not-pregnant) had 39 longer median days not-pregnant compared with LE < +++ (HR = 0.66; 95 % C.I. 0.45 -0.97; *P* = 0.03). Body condition score (HR = 1.52; 95 % C.I. 1.16 – 1.20; *P* = 0.002) and days postpartum at first-insemination (HR = 0.98; 95 % C.I. 0.97 – 0.99; *P* = 0.003) were retained in the final model. In primiparous cows, the HR for LE was not significant (*P* > 0.20) at the highest cutoff with any combination of covariates. The pregnancy survival curves for LE ≥+++ effect stratified by parity are shown in **Figure 3.1**.

Protein concentration was not significant at any cutoff point, combined or stratified by parity (*P* > 0.20) on calving to conception interval.

A significant effect on hazard of pregnancy was found for pH ≥ 7.0 and the interaction between pH ≥ 7.0 and parity was significant (*P* = 0.04). Stratification by parity revealed the effects to be limited once again to multiparous cows where the median calving-to-conception interval increased from 111.5 d in pH < 7 to 150.5 d in pH ≥ 7.0 (HR = 0.68; 95 % C.I. 0.50 – 0.92; *P* = 0.01) with body condition score (HR = 1.50; 95 % C.I. 1.14 – 1.96; *P* = 0.003) and days postpartum at first-insemination (HR = 0.98; 95 % C.I. 0.97 – 0.99; *P* = 0.001) also retained in the final model. In primiparous cows, pH was not significant (*P* > 0.20) at any cutoff and covariate combination.

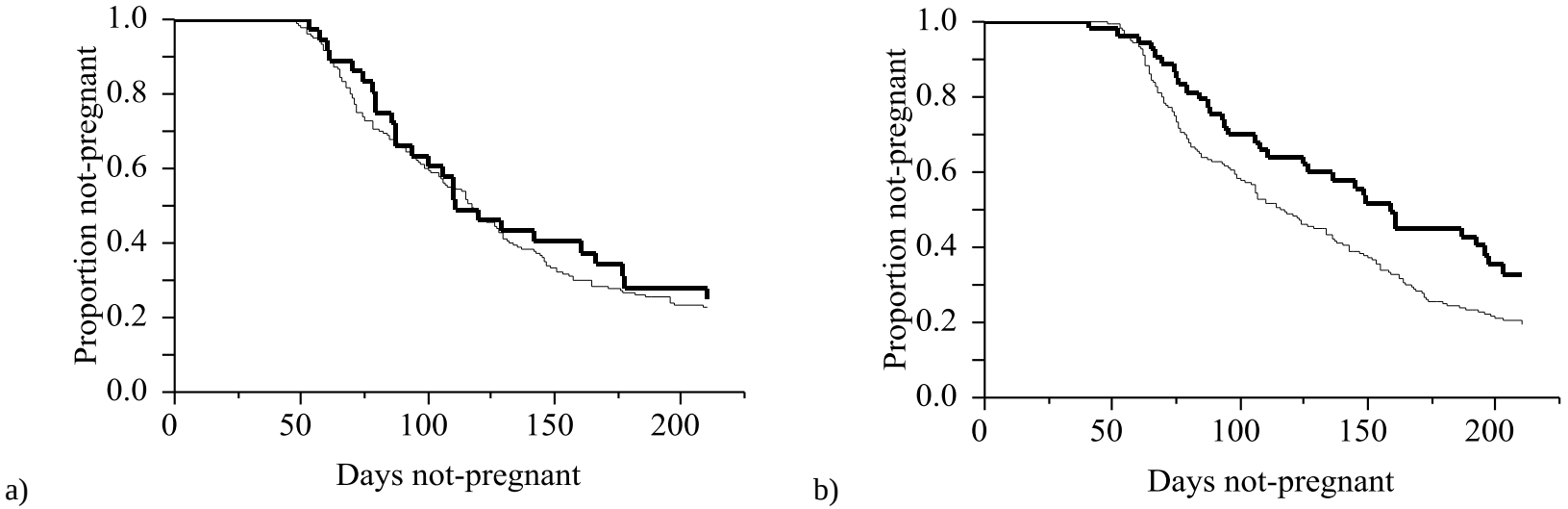

Figure 3.1.

Kaplan-Meier survival curves for LE ≥+++ (thick line) effects on calving-to-conception interval. a) Primiparous cows with LE ≥+++ did not show impaired reproductive performance compared against cows with LE <+++ (median days not-pregnant 111 and 117d; $P = 0.57$) ; b) multiparous cows with LE ≥+++ had longer median days not-pregnant (154 d) compared with cows with LE <+++ (116 d) ($P = 0.02$).

Factors associated with a reagent strip test

Cytologically diagnosed endometritis was significantly associated with the reagent strip tests and was retained in the final models of LE (OR = 4.49; 95% C.I. 3.07 -6.56; $P < 0.0001$), protein (OR = 1.84; 95% C.I. 1.26 – 2.67; $P = 0.0015$) and pH (OR = 2.67; 95% C.I. 1.88 -3.79; $P < 0.0001$). Herd was significant in all of the final models. The final model for LE did not retain any other fixed variables whereas the final model for pH retained parity (Primiparous OR = 1.79; 95% C.I. 1.29 – 2.49. $P = 0.001$) and in the final model for protein, retained placenta was retained (OR = 1.94; 95% C.I. 1.02 – 3.69. $P = 0.045$).

Reagent test used in combination

Although protein was associated with cytological endometritis, the use of this test in combination did not improve the overall performance of the other reagent strip tests. The cutoff point of ≥ +++ for LE in combination with pH ≥ 7.0 was found to have the best performance. Using this combination of cutoff points, NPV was 75.6 % (394/521) while the PPV was 69.0 % (29/42) with 18.6 % Se and 96.8 % Sp. A closer examination of the combined performance of LE and pH at each category to diagnose cytological endometritis found the test to be much more accurate at the high and low end while cows that had LE = ++ but pH ≥ 7.0 and cows that had LE = +++ but pH < 7.0 could not reliably be assigned an endometritis classification. Therefore the results were stratified into the positive group (LE ≥ ++ and pH ≥ 7.0), negative group (LE < ++ and pH < 7.0) and the undetermined group (LE = ++ but pH ≥ 7.0; or LE = +++ but pH < 7.0). The PPV for positive group was 69.0 % and the NPV for the negative group was 82.5% however there was a large group of undetermined cows (20.6% of cows).

Kaplan-Meier survival curves for calving-to-conception interval stratified by parity using combined LE and pH are shown in **Figure 3.2**. Once again reproductive impairment was restricted to multiparous cows where combination positive cows had 184 median days not-pregnant while undetermined cows had 129 median days not-pregnant and combination negative cows had 113 median days not-pregnant ($P < 0.001$).

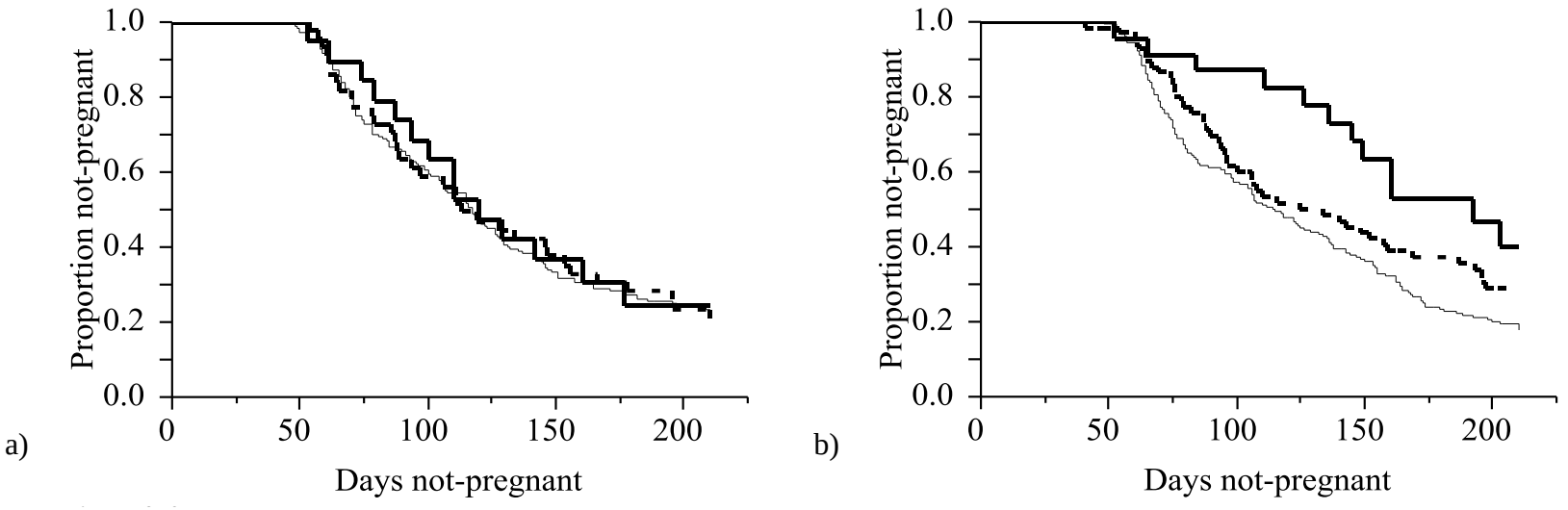

Figure 3.2. Kaplan-Meier survival curves for combining LE and pH results on calving-to-conception interval. a) Primiparous cows in the positive group (LE ≥+++ and pH ≥7.0; thick solid line) were not different from the undetermined group (LE = ++ and pH ≥7.0 or LE ≥+++ and pH <7.0; interrupted thick line) and negative group (LE <++ and pH <7.0; thin line). b) Multiparous cows in the positive group (184 median days not-pregnant) were significantly different from the undetermined group (129 median days not-pregnant) and negative group (113 median days not-pregnant; $P < 0.001$).

DISCUSSION

Reagent strip results of LE, protein and pH reagent strip were highly associated with cytological endometritis. However, the performance of LE in this study was not as accurate as described by Santos et al. [8]. There are three important differences between Santos et al. and the present study. First, the Santos study set the LE cutoff point at + and endometritis cutoff point at 5.5 % neutrophils. The analysis was repeated using data from this study at these cutoff points and found the LE test to have a Se of 48.8 % and Sp of 73.3 %, which was still lower than the 83 % Se and 94 % Sp reported by Santos et al. Secondly, cows were sampled between 1 and 7 wk postpartum by Santos et al while cows were sampled later postpartum between 40 and 60 d in the present study. Cows in the early postpartum period tend to have very high proportions of neutrophils in endometrial cytology [2], which may have improved the performance of the reagent strip test. Finally, the uterine lavage samples for the current study were only tested at the laboratory and the time interval between sampling and testing was large in some cases as the farms sampled were an average 3 h drive from the laboratory. The reactivity of LE in human urine with reagent strip is decreased in approximately 25% of samples after 24 h of refrigeration [11] increasing FN results. In the present study, the FN results remained low for LE despite the long transport time.

Inflammatory conditions in cattle have been reported to cause an increase in the pH of fluid excretions [9, 10]. In the present study, the uterine fluid pH was increased in cows with endometritis relative to normal cows and the correlation with endometritis was even higher than the LE test. The optimal cutoff point of pH $\geq$ 7.0 was similar to the recommended cutoff point for semen from bulls with seminal vesiculitis [10]. To our knowledge this is the first report of increased pH in uterine fluid of cows with endometritis. The Se and Sp of pH to diagnose endometritis at the

optimal cutoff point ≥ 7.0 was still relatively poor at only 44.9 % and 78.4 %, respectively.

The protein reagent strip test had the weakest association with cytological endometritis and was not predictive of future reproductive performance. Storage of refrigerated human urine samples for 24 h increased the FP results in reagent strips [11]. The observed PPV for the protein test was low even at the highest cut-off point (43.7 %) which may be attributed to the long transport time between sample collection and testing.

Multistix® 10 SG is a reagent strip designed as a rapid test for human urinalysis. The LE reagent strip is a highly sensitive test for human pyuria and is an excellent screening test [12]. Conversely, the LE reagent strip test is not very sensitive and is not recommended as a screening test for pyuria in small animal veterinary medicine [13, 14]. The different source of samples tested could affect the performance of the test. There are no published studies evaluating the repeatability of LE, protein or pH on the Multistix® 10 SG reagent strip. The LE and protein reagent strips were not designed for uterine lavage and the available increments of reference results at the critical concentrations for endometritis are too large. The overall prevalence of endometritis by cytology was 27.7 % but the prevalence at the optimal cutoff points for LE and protein was 56.1 % and 48.1 % respectively, which is a gross overestimation. The next category for LE and protein grossly underestimated the prevalence at 16.5 % and 12.6 % respectively. At the optimal cut-off point for pH, the overall prevalence was 28.1 % which is close to the cytologically-derived prevalence in this study. The categorical increment for pH around the critical point for cytological endometritis was 0.5 which appears to be narrow enough while the available categories for LE and protein do not appear to have sufficient resolution for optimal performance. Combining LE and pH results can improve the PPV to 69.0 %

and the NPV to 82.5 % however; there will be a group of undetermined cows (20.6 %).

In summary, reagent strip results were significantly associated with cytological endometritis and predicted poor reproductive performance. However, the Se and Sp of reagent strip tests were relatively poor. Modification of the test strips to optimize diagnostic categories for bovine endometritis seems to offer the potential for an accurate and convenient cow-side diagnostic tool.

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CHAPTER 4

EFFECTS OF DIAGNOSTIC LOW-VOLUME UTERINE LAVAGE SHORTLY BEFORE FIRST SERVICE ON REPRODUCTIVE PERFORMANCE, CULLING AND MILK PRODUCTION

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ABSTRACT

The objective of this study was to evaluate if the process of uterine sampling by low-volume uterine lavage, performed shortly before the end of the voluntary waiting period, had any effect on reproductive performance, milk production or culling. Approximately 20 cows between 40 and 60 d postpartum were sampled from 38 herds. Non-sampled cows were all the remaining cows that were between 40 and 60 d postpartum present at the time of sampling in that herd. Uterine lavage samples were obtained from 752 cows and there were 2,252 non-sampled cows in the reference cohort. There was an interaction between parity and sampling for first-service conception, calving to conception interval and milk production. When stratified by parity, there was a tendency for impaired reproductive performance in sampled primiparous cows compared with non-sampled primiparous cows (Odds Ratio for pregnancy to first-service = 0.78 (95 % C.I. 0.58 – 1.04). The Hazard Ratio for pregnancy in sampled primiparous cows was 0.89 (95 % C.I. 0.77 – 1.04) compared with non-sampled primiparous cows. Sampling did not have any effect on first-service conception rate (Odds Ratio for pregnancy OR for pregnancy = 1.03; 95 % C.I. 0.80 – 1.33) or calving-to conception interval in multiparous cows (Hazard Ratio = 1.04; 95 % C.I. 0.91 - 1.18). Sampling did not affect culling risk (Hazard Ratio HR = 0.92; 95 % C.I. 0.77 – 1.11) after accounting for covariates. After stratification by parity, milk production was not affected by sampling except in fourth- and fifth- parity cows where sampled cows produced more milk than non-sampled cows after controlling for first test-day milk production and days-postpartum at first test-day. In conclusion, sampling by low-volume uterine lavage did not have significant detrimental effects on reproduction, culling or milk production. However, there was a tendency for lower first-service conception in sampled primiparous cows but the

procedure appears to be benign in multiparous cows.

INTRODUCTION

Endometritis is a common uterine health disorder that impairs fertility in postpartum cows [1-7]. Affected cows, do not consistently display external signs making the diagnosis of this condition difficult [2,3]. Evaluation of diagnostic techniques to detect subclinical inflammation of the uterus found cytological evaluation of uterine samples to be superior to transrectal ultrasound examination for fluid accumulation in the uterine lumen or cervical diameter [4,5] when correlating with reproductive outcomes. Dubuc et al. [2] proposed the presence of subclinical inflammation of the uterus by cytology to be cytological endometritis regardless of the presence of purulent material in the vagina, which was part of the definition of subclinical endometritis proposed by Sheldon et al. [3], as the inflammation in the uterus and presence of purulent material in the vagina are different conditions with additive reproductive consequences. Low-volume uterine lavage is one of the methods used to obtain uterine samples for diagnosis of endometritis by cytology in cows [1,5]. The other sampling method used to obtain uterine samples for cytology is the cytobrush method. The cytobrush method performed 4 h post AI was found to have no detrimental effect on first-service conception rate [8] but there is no data on the effects of sampling before the first-service at unspecified stages of the estrus cycle, which is the predominant time of sampling in most studies [1-7], on subsequent reproductive performance, milk production or culling. The diagnosis of cytological endometritis was only associated with impaired reproductive performance if taken after 35 d post partum and the association with reproductive impairment was stronger when uterine samples were collected later at 49 d postpartum [8]. Therefore, uterine

samples collected shortly before the end of the voluntary waiting period would be more valuable however this may leave the sampled cow with less time to recover from potential damage or contamination before being bred. In addition to the sampling procedure itself, collection of uterine samples requires some handling of cows and this may contribute to the overall effects of sampling.

The objective of this study was to determine if diagnostic uterine sample collection by low-volume uterine lavage in lactating postpartum dairy cows shortly before the end of the voluntary waiting period affected reproduction, culling and milk production.

MATERIALS AND METHODS

Study subjects

Low-volume uterine lavage samples were collected as part of a separate study [6]. A cross sectional sampling design was used within each herd and cows were followed up in a cohort study. Cows were sampled once and follow-up data obtained six months after sampling to determine reproductive and milk production performance. Eligibility criteria for sampling were: between 40 and 60 d postpartum, neither inseminated nor within two d of the end of the voluntary waiting period and apparently healthy by cursory visual evaluation of attitude and no obvious signs of systemic illness, and without vaginal discharge by external visual examination. Approximately 20 cows were sampled from each herd and herds were selected based on a convenience sample of: producers willing to participate in the study, who used DairyComp305 (Valley Agricultural Software, Tulare, CA) as the herd record management software and who had a herd size of at least 400 milking cows. The large herd size was required to ensure at least 20 cows were available within the sampling

window at any given time. About one third of the samples were obtained from primiparous cows to reflect normal herd demographics. All sampled herds used timed-artificial insemination with slightly varying use of visual and activity heat detection.

The reference population (non-sampled) cows used for the analysis were all herd-mates also between 40 and 60 d postpartum, present in the herd at the time of sampling and not exposed to sampling by uterine lavage. Additional criteria for non-sampled cows were: not bred before 40 d postpartum and not designated 'Do-Not-Breed' (DNB) before 40 d postpartum. The proportion of cows with and without cytological endometritis in the reference population was assumed to be similar to the sampled population.

Animal handling and sample collection

Initial lists of eligible cows were obtained from dairy records at the time of sampling. Eligible cows were identified and marked using commercial livestock marker and headlocks were set where available. Marked cows were restrained in headlocks or in stalls by convenience and sampled. The perineum was cleansed using paper towels and a 63.5 cm sterile flex tip infusion pipette (Exodus Breeders Corporation, York, PA, USA) was introduced through the vagina and cervix into the uterine body. Twenty mL of sterile isotonic saline was infused into the uterus using a sterile syringe through the pipette, the uterus was massaged by palpation per rectum and the fluid was recovered into the same syringe. Approximately 5 – 8 mL of fluid was recovered and the remainder of the fluid was left to be evacuated through the cervix. Sampled cows were marked to prevent double sampling. This process was repeated until approximately 20 samples were collected from each herd.

Data management and statistical analysis

Reproductive, culling and milk production data were obtained from follow-up herd records. The follow-up period for the study was until 210 d postpartum. The herd managers were unaware of which cows were sampled and thus the cows were not subjected to any differential treatment. The assumption was that the unobserved risk factors would be equally distributed between the sampled and non-sampled cows within demographic groups in each herd. Primiparous cows were usually housed separately from multiparous cows at the time of sample collection and therefore parity was tested in statistical model building. To account for the unmeasured error at the cow-level that is clustered at the herd-level, "Herd" as a random effect variable was included in all analyses. Fixed effect variables were considered significant and retained in model building if $P < 0.05$. Two-way interactions were considered significant if $P < 0.20$ and the analyses were repeated stratified by the interacting variables.

To determine the effect of low volume uterine lavage on reproductive outcome, conception rate to the first-service and hazard of pregnancy up to 210 d postpartum was examined. Cows that were not pregnant by 210 d postpartum were right censored. Conception rate to first-service was evaluated using categorical regression model in the GLIMMIX procedure of SAS version 9.2 (SAS Institute, Cary, NC, USA) with herd included as a random effect variable. Sampling (sampled or non-sampled) was forced into the model. Covariates tested in model building were: days-postpartum at first-service (continuous), parity (primiparous and multiparous) and two-way interactions. Kaplan-Meier survival curves for calving-to-conception interval were produced and evaluated using JMP version 8 (SAS Institute, Cary, NC,

USA). Hazard of pregnancy was evaluated by use of the Cox proportional hazards model including herd as a shared frailty variable and was calculated using Stata/IC version 10 (StataCorp, College Station, TX, USA). Cows were censored if they were designated DNB, were culled or died, or were not pregnant by the end of the follow-up period of 210 d postpartum. Sampling (sampled or non-sampled) was forced into the model and the covariates tested were the same as for conception to first-service analysis.

The effect of low volume uterine lavage on risk (hazard) of culling was evaluated using the Cox proportional hazards as before, including herd as a shared frailty variable using Stata/IC version10. Kaplan-Meier survival curves were produced using JMP version 8 for calving-to-culling interval. Cows were right censored if not culled or dead by 210 d postpartum. Sampling was forced into the model. Parity (primiparous or multiparous) and interaction with sampling were tested as covariates.

Sampling effect on milk production was evaluated using test-day milk production in a repeated-measures analysis using PROC MIXED of SAS version 9.2. Test-number was assigned based on days-postpartum at test-day in increments of 30 d. As the end of the follow-up period was 210 d postpartum, the possible test numbers were from Test1 to Test7. As the cows were only eligible to be sampled after the first 40 d, Test1 or first test-day milk was not considered as a possible outcome. Instead, it was tested in the model as a covariate. Other variables tested as covariates in the model were: days-postpartum at first-test (continuous), test-number (categorical 2 – 7), days-postpartum at test-day (continuous), parity (primiparous, second parity and third or greater parity) and interactions. Continuous data was transformed as necessary to maintain homoskedasticity of Pearson and scaled residuals.

RESULTS

Pregnancy to first-service

There were 705 sampled and 1,992 non-sampled cows in the reference population that were bred at least once. The average time from sampling to first-service was 19.4 d (SEM = 0.4) and the sampling to first-service interval was similar between primiparous and multiparous cows. In the analysis of pregnancy to first-service, the interaction between parity and sampling group had $P = 0.12$ and therefore the analyses were stratified by parity.

In primiparous cows, days-postpartum at first-service was not significant ($P = 0.89$) and was dropped from the model. There was a tendency for sampled primiparous cows (88/282; 31.2 %) to have impaired conception to first-service compared with non-sampled cows (289/791, 36.5 %) with Odds Ratio (OR) for pregnancy = 0.78 (95 % C.I. 0.58 – 1.04; $P = 0.09$). Herd was significant as a random variable ($P = 0.04$).

In multiparous cows, parity within multiparous cows ($P = 0.53$) and days-postpartum at first-service were not significant ($P = 0.27$) and dropped from the model. However, there was no effect of sampling on conception to first-service (OR for pregnancy = 1.03; 95 % C.I. 0.80 – 1.33; $P = 0.82$). The conception rate to first-service was 123/423 (29.1 %) in sampled cows and 337/1,201 (28.1 %) in non-sampled multiparous cows respectively (**Table 4.1)**.

Table 4.1. Final logistic model for the effect of sampling on first-service conception rate stratified by parity. Sampling was forced into the model and herd included as a random variable.

		OR/Estimate	95 % C.I.		*P*
Parity	Variable		Lower	Upper	
Primiparous	Sampled	0.78	0.58	1.04	0.09
Multiparous	Sampled	1.03	0.80	1.33	0.82

Calving-to-conception interval

There were 752 sampled and 2,252 non-sampled cows used for this analysis. There was an interaction between parity and sampling group on hazard of pregnancy ($P = 0.09$) and the analyses were stratified by parity groups. In primiparous cows, sampled cows had HR for pregnancy = 0.89 (95 % C.I. 0.77 – 1.04; $P = 0.18$) with days-postpartum at first-service also retained in the model (HR = 0.99; SEM = 0.001; $P < 0.001$). The Kaplan-Meier survival curve for calving-to-conception interval in primiparous cows is shown in **Figure 4.1 panel A**.

In multiparous cows, sampled cows had HR for pregnancy = 1.04 (95 % C.I. 0.91 -1.18; $P = 0.58$) compared with non-sampled cows. Days-postpartum at first-service and parity within multiparous cows were significant ($P < 0.001$) and retained in the model. The effect of sampling was not significant when examined by parity within multiparous cows. The Kaplan-Meier survival curves for sampling effect on calving-to-conception interval in multiparous cows are shown in **Figure 4.1 panel B**.

Culling

There were 752 sampled and 2,252 non-sampled cows used for this analysis. Parity was retained in the final model ($P < 0.001$). Uterine lavage sampling did not affect culling (HR = 0.92; 95 % C.I. 0.77 – 1.11. $P = 0.56$). Herd was significant as a random variable ($P < 0.001$). The Kaplan-Meier survival curve for time to culling is shown in **Figure 4.2**.

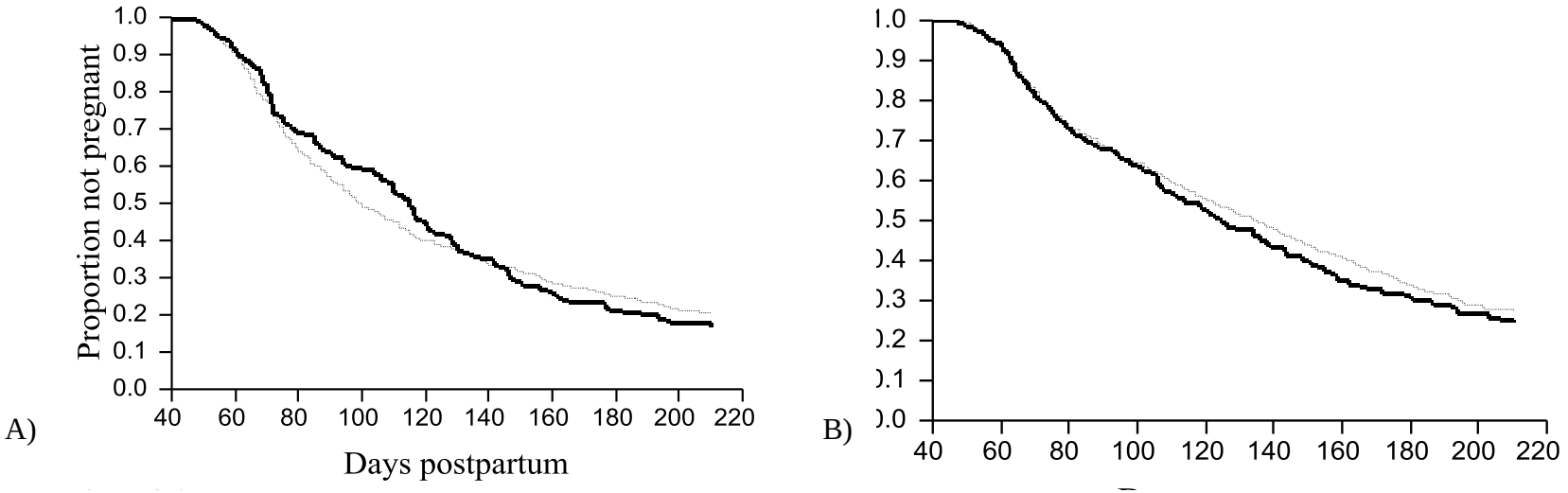

Figure 4.1. Kaplan-Meier survival curve for calving-to-conception interval in a) sampled (thick line, n = 285) and non-sampled (thin line, n = 1,112) primiparous cows and b) sampled (thick line, n = 467) and non-sampled (thin line, n = 1,892) multiparous cows. Survival curves were not significantly different.

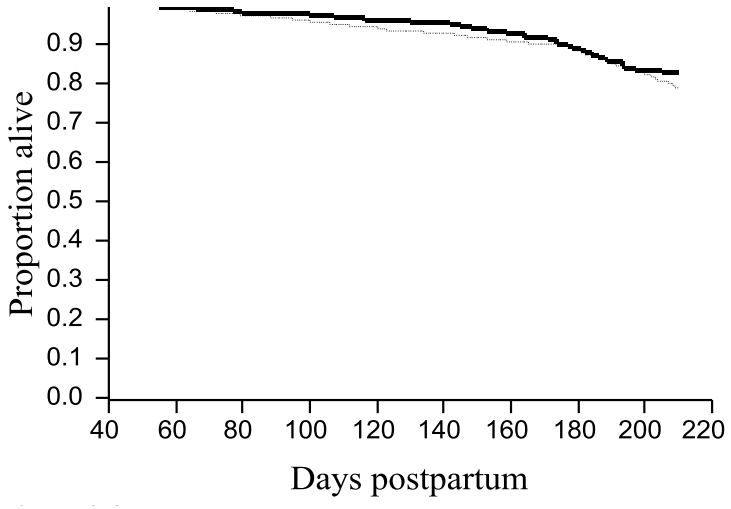

Figure 4.2. Kaplan-Meier survival curve for time-to-culling grouped by sampled cows (thick line, n = 752) and non-sampled cows (thin line, n = 2,252) was not significantly different.

Milk production

There were 530 sampled and 1,532 non-sampled cows from 33 herds used in this analysis. Several herds did not have the first test-day milk until after 30 d postpartum and were dropped. Days-postpartum at test-day was not significant and was dropped from the milk analysis. There was an interaction between parity and sampling (P = 0.01) and thus the analysis was stratified by parity.

Sampling did not affect milk production in primiparous (P = 0.61) cows, second-parity cows (P = 0.76), third parity cows (P = 0.47), and sixth-or-greater parity cows (P = 0.91). However, sampled forth-parity cows produced 3.6 kg/d, SEM 1.3 (P = 0.005) more milk and fifth-parity cows produced 4.7 kg/d, SEM=2.3 (P = 0.03) more milk than non-sampled cows after controlling for first-test-day milk production, test number, and days-postpartum at first test-day. The interaction between Test number and Sampling was not significant (P > 0.20), overall or within parity groups.

DISCUSSION

Sampled primiparous cows had a tendency for lower conception rate to first-service (P = 0.09) compared with non-sampled cows but this was not seen in multiparous cows. One important consideration is that non-sampled cows were not subjected to mock sampling or any kind of handling comparable to that experienced by the sampled group. Anecdotally, primiparous cows appeared more nervous when being marked and sampled compared with multiparous cows. It is impossible to determine how much of the observed effect was caused by the stress associated with handling and how much of the observed effect was caused by the sampling procedure itself. There were no significant detrimental effects of sampling on long-term reproductive performance.

The purpose of sample collection was to diagnose cytological endometritis but

the disease status was not associated with impaired reproductive performance in primiparous cows [6]. This makes sampling of primiparous cows not only potentially detrimental to first-service conception rates but the information obtained was of limited value due to the poor association with economically important outcomes. Conversely, sampling of multiparous cows did not affect reproductive outcomes but the information obtained (endometritis status) was associated with impaired reproductive performance [6]. Future studies on cytological endometritis might be directed towards multiparous cow to maximize potential benefits and minimize potential detrimental effects.

Sampling did not significantly affect culling risk in any parity group of cows. The high-risk period for culling early postpartum occurred before the eligibility window, and the right-censoring at 210 d postpartum is earlier than most culling for reproductive failure. This resulted in a generally low culling rate for the eligible cows in the study period both in the sampled as non-sampled group.

Culling risk increases with impaired reproduction [9]. Cytological and subclinical endometritis has been associated with impaired reproductive performance [1-7] however cytological endometritis was not shown to affect culling risk by 300 d postpartum [10]. In the present study, cytological endometritis status did not increase culling risk (data not shown, $P = 0.20$) by 210 d postpartum in any parity group despite affected primiparous cows showing impaired reproductive performance [6].

Milk production was higher ($P = 0.03$) overall in sampled cows compared with non-sampled cows however there was a significant interaction between sampling effect and parity. Stratified by parity, the increased milk production was only observed in fourth- and fifth- parity cows. The interaction between sampling and test-day number was not significant even when stratified by parity in any parity group. First test-day milk production was similar between sampled and non-sampled cows after controlling for days-postpartum at first test-day and parity. The primary concern was for potential economic loss to the producers from sampling and there were no detrimental effects of

sampling on milk production observed in the present study.

The association between cytological endometritis and milk production was different between primiparous and multiparous cows [11,12] and the interaction in the present study was $P = 0.11$ (data not shown). In primiparous cows, milk production was numerically higher in cows with cytological endometritis although the difference was not significant [11] and this was also observed in the present study (data not shown; $P > 0.20$). In multiparous cows with cytological endometritis, the milk production was lower than unaffected cows and some studies have found significant differences at specific time point [11,12] while others [10] found no significant differences. First test-day milk production was significantly lower in multiparous cows with cytological endometritis compared to unaffected cows after accounting for days-postpartum at first test-day and herd as a random variable (data not shown; $P = 0.002$).

The only other study evaluating the effects of sample collection for uterine cytology in cattle was Kaufmann et al. [8] which found no detrimental effects of sampling using the cytobrush method shortly after AI on first-service conception. Sampling in the present study was performed prior to AI at a fixed time postpartum which was closer to the sampling schedule in other studies [1,2,4-7,10-12]. The power of the study was good and liberal *P*-values were used to minimize type-II error which was important in this study.

In conclusion, sampling by low-volume uterine lavage did not have significant detrimental effects on reproduction, culling or milk production. However, there was a tendency for lower first-service conception in sampled primiparous cows but not in multiparous cows.

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CHAPTER 5

METABOLIC AND ENDOCRINE DIFFERENCES BETWEEN COWS THAT DO AND DO NOT OVULATE THE FIRST POSTPARYM DOMINANT FOLLICLE

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ABSTRACT

Most dairy cows develop the first dominant follicle postpartum within 2 weeks after calving but about 40% of these do not produce estradiol and do not ovulate despite having normal ultrasound appearance and growth. This study aimed to identify difference in follicular fluid steroid hormone profile, endocrine and metabolic status of cows that ovulate the first dominant follicle postpartum and those that do not. Prediction of the fate of the first dominant follicle postpartum of multiparous Holstein dairy cows (n=59) was validated and performed by using follicle growth characteristics and circulating estradiol concentrations. Follicular fluid was aspirated in a subset of cows for steroid hormone analysis and follicular fluid estradiol and androsteinedione concentrations were lower in non-ovulatory follicles. Luteinizing hormone pulse frequency was also lower in non-ovulatory cows and together with the low follicular fluid androsteinedione levels show the theca cells function to be impaired. Prepartum and early postpartum estradiol, progesterone, and insulin-like growth factor-1 profiles were similar between follicle fate groups. Non-ovulatory cows had lower feed intake resulting in lower energy state compared with ovulatory cows. Glucose tolerance test on the day of follicular fluid aspiration showed non-ovulatory cows have slower glucose clearance and lower insulin response compared with ovulatory cows. This study describes a novel follicle fate prediction method for the first dominant follicle postpartum in dairy cows and report for the first time the steroid hormone profile of the non-ovulatory follicle which shows that theca cell function is impaired.

INTRODUCTION

Early resumption of ovarian cyclicity postpartum in dairy cows has been associated with improved subsequent reproductive performance [1-3]. Follicular activity ceases in late gestation and resumes shortly after calving when the clearance of

pregnancy hormones induces follicle stimulating hormone (FSH) secretions [4]. Most cows develop a cohort of ovarian follicles with a dominant follicle emerging within the first two weeks postpartum [4-6]. However, only 40% of these first dominant follicles ovulate. In previous studies [5, 7], cows that ovulate the first dominant follicle postpartum exhibited the typical preovulatory rise in circulating estradiol, while the majority of cows that did not ovulate, did not have high circulating estradiol levels.

The production of estradiol by ovarian follicles requires functional theca and granulosa cells [8, 9]. Theca cells metabolize cholesterol to androgens under luteinizing hormone (LH) stimulation [10]. Granulosa cells then aromatize androgens to estradiol under FSH stimulation [11]. This is the two-cell two-gonadotropin model of steroidogenesis [12]. By measuring the intermediate hormone concentrations in follicular fluid, this study aimed to determine if the failure of non-ovulatory follicles to produce high circulating estradiol levels was due to impairment of the theca interna or the granulosa cells.

Energy balance is an important component in determining the fate of the first dominant follicle postpartum [13]. High producing dairy cows enter a state of negative energy balance after parturition which results in reduced pulsatile gonadotropin releasing hormone (GnRH) and thus LH release [11, 14], and increases the sensitivity of hypothalamic negative feedback by estradiol that could lead to ovulatory failure [5]. In addition, reduced circulating levels of insulin, insulin-like-growth factor-1 (IGF-1) and glucose, as well as increased concentrations of β-hydroxybutyrate acid (BHBA) and non-esterified fatty acids (NEFA) occurring during postpartum negative energy balance, are all associated with impairment of estradiol production and delay to first ovulation [15-17]. Insulin resistance occurs in late pregnancy and early lactation in high producing dairy cows [15, 16, 18]. The inability of insulin-resistant cows to promote lipogenesis and prevent lipolysis [19] result in increased circulating and follicular fluid concentrations of NEFA and BHBA which has been associated with impaired follicle

function [20, 21]. Insulin directly increases steroidogenic response of granulosa and theca cells to gonadotropin stimulation in vitro [22]. Therefore, characterizing the energy balance, endocrine, metabolic and insulin resistance status during the prepartum and early postpartum period will allow better understanding of the physiological state of the cows that fail to ovulate.

A novel prediction method to determine the fate of the first dominant follicle postpartum was validated and used to obtain follicle fluid from ovulatory and non-ovulatory follicles. The goal of this study was to determine the level of steroidogenic dysfunction in non-ovulatory cows to the granulosa or theca cell by evaluating the steroid hormone profiles in follicular fluid. In addition, the difference in metabolic and endocrine status was evaluated between cows that do and do not ovulate the first dominant follicle postpartum.

MATERIALS AND METHODS

Animals

All animal procedures were approved by the Cornell University Institutional Animal Care and Use Committee. Handling of cows was in accordance with the U.S. Department of Agriculture's "Guide for the Care and Use of Agricultural Animals in Agriculture Research and Teaching" [23]. Experimental animals were obtained, and the study was conducted, at the Cornell Teaching and Research Dairy unit. Multiparous pregnant Holstein dairy cows (n = 59) were enrolled into the study 28 days prior to their expected calving date. Cows were housed individually in tie-stalls with free access to water and were individually fed with total-mixed-ration formulated to meet or exceed all National Research Council (NRC) 2001 [24] requirements appropriate to the stage of lactation. After calving, cows were milked three times a day at 0030, 0730 and 1430 h. Seven cows were dropped from the study due to diseases which were: displaced abomasum (two cows), dystocia requiring veterinary intervention (one cow), and

chronic fever (≥ 39.5°C for ≥ 3 days) that did not respond to antibiotics (four cows).

All cows were subjected to the same sampling and measuring schedule until classified as ovulatory or non-ovulatory. Blood samples were collected from the coccygeal vein into heparinized vacutainer tubes three times a week (Monday, Wednesday and Friday) from 28 to 10 days before the due date then daily until three days after the group assignment. Plasma was separated from cellular components by centrifugation (4°C, 20 min at 1,000 g) then collected and stored at -20°C until assayed for estradiol, progesterone, insulin, IGF-1, NEFA, BHBA, and glucose concentrations.

A glucose tolerance test (GTT) was performed prepartum at 10 days before due date and another GTT performed postpartum on the day after follicle fate prediction. A jugular catheter was placed one day before the GTT and cows were fasted for three hours prior to glucose infusion. Glucose was administered as a bolus of 0.25 g/kg body weight in the form of 50% dextrose through the jugular catheter. Heparinized saline was used to flush the jugular catheter after glucose infusion and after each sample collection. Blood samples were collected at -15, -1, 1, 3, 6, 9, 12, 15, 20, 25, 30, 60, 90, 120 and 150 min after glucose infusion. Samples were analyzed for glucose and insulin.

To determine LH pulse frequency, blood samples were collected every 10 min for 6 h through the jugular catheter on the day of the postpartum GTT and analyzed for LH levels.

Follicular fluid was collected from the first dominant follicle postpartum the day after group assignment by ultrasound-guided needle aspiration. A 5 MHz convex ultrasound probe (Aloka Inc, Wallingford, CT) in an aspiration case was introduced per vagina and the ovary was placed on the probe by transrectal manipulation. The aspiration needle was introduced into the follicle and the follicular fluid was collected in two fractions to minimize blood contamination. The first fraction collected the first 500 μL and the second fraction collected the remainder of the follicular fluid. Follicular fluid samples were centrifuged (4°C, 2 min at 2,000 g) and the supernatant was stored at

-20°C until assayed for estradiol, progesterone, androsteinedione, IGF-1 and NEFA.

Group assignment

Follicle fate must be predicted to allow collection and analysis of follicular fluid. To determine suitable criterion to predict follicle fate, data from previous studies characterizing the follicle growth and circulating estradiol concentrations of the first dominant follicle postpartum [5, 7] was used. In the previous studies, all ovulatory cows had a rise of circulating estradiol of ≥ 2 pg/mL at least one day before ovulation and all cows had a dominant follicle of ≥ 10 mm in diameter present. Cows that had follicles that failed to grow after reaching a diameter of at least 10 mm, and cows that had follicles that regressed did not ovulate. In addition, cows that had follicles that reached ≥ 15 mm in diameter and did not have a rise in circulatory estradiol by one day after reaching this size threshold also did not ovulate.

For the present study, ovarian follicular activity was examined daily starting from 7 days postpartum using a 7.5 MHz linear ultrasound probe (Aloka Inc, Wallingford CT). Follicles were measured using internal calipers and mapped on the ovary daily to determine follicular growth. Once a follicle grew to 10 mm in diameter, it was considered the first dominant follicle postpartum. The criterions for follicle fate group assignment were: 1) Cows were assigned to the ovulatory group if circulating estradiol was ≥ 2 pg/mL with a follicle > 10 mm in diameter. 2) Cows were assigned to the non-ovulatory group if: the diameter of the first dominant follicle reached 15 mm; and 3) cows were also assigned to the non-ovulatory group if circulating estradiol did not reach a concentration of ≥ 2 pg/mL by the following day; or if the follicle failed to grow or regresses in size before achieving 15 mm in diameter.

To validate the classification of ovulatory group, a subset of cows (n = 9) was not aspirated and followed up until 30 days postpartum. These control cows were subjected to the same sampling protocol except the first dominant follicle postpartum

was not aspirated the day after group assignment. Instead, these control ovulatory and control non-ovulatory cows were continued to be examined until 30 days postpartum by transrectal ultrasound and circulating hormone concentrations to determine if the first dominant follicle postpartum ovulated, and formed a competent corpus luteum. In addition, a subset (n = 31) of ovulatory and non-ovulatory cows that had the first dominant follicle postpartum aspirated, were monitored with transrectal ultrasound examination of the ovaries three times a week until 30 days postpartum to determine if the subsequent dominant follicle ovulated.

Hormone and metabolite assays

Plasma and follicular fluid estradiol concentration was measured by RIA (Serono-Maia, Cortland Manor, NY) as previously described [25] with the following modifications: plasma estradiol was extracted from 200 μL of sample with 3 mL of benzene: toluene (2:1 vol/vol) solvent, and follicular fluid was diluted to 1:1000 for cows with high circulating estradiol while follicular fluid from cows with low circulating estradiol was diluted to 1:250. Six follicular fluid samples from cows with low circulating estradiol were diluted to 1:500 to be within the standard curve range of the estradiol assay. Plasma and follicular fluid progesterone were measured by RIA as previously described [26] with the following exceptions: progesterone for iodination and standards used was supplied from MP Biomedical Diagnostic; Orangeburg, NY; follicular fluid progesterone was not extracted and was diluted to ensure the tested concentrations fell within the standard curve range. Total IGF-1 concentrations in plasma and follicular fluid were determined using RIA as previously described [15] with the exception that the source human IGF-1 for iodination and standards were obtained from Gropep Limited, Australia. Insulin concentrations in plasma including GTT samples were determined using Porcine Insulin RIA kits (Millipore, Billerica, MA) validated in our lab. Plasma LH concentrations were determined by RIA as previously

described [25] with rabbit antibovine LH (#R 16) antisera was a gift from R.B. Staigmiller. Follicular fluid androsteinedione concentrations were determined by RIA as previously described [27] using serially diluted samples of 1:5 to 1:50. Plasma BHBA (kit 310-UV Sigma Diagnostics, St Louis, MO), NEFA (NEFA-C kit, Wako chemicals, Richmond, VA) and glucose (kit 510A Sigma Diagnostics, St Louis, MO) concentrations were measured using commercial enzymatic colorimetric assays validated in our lab. Inter and intra assay CVs for estradiol, progesterone, IGF-1, insulin, LH, androsteinedione, BHBA, NEFA and glucose were: 9.5% and 5.9%, 10.9% and 7.5%, 13.9% and 6.1%, 9.4% and 6.7%, 7.0% and 3.6%, 4.9% and 4.7%, 5.5 % and 4.2%, 3.4% and 2.3%, 9.4% and 3.9% respectively.

Daily energy balance calculation

Scale body weight and the average body condition score [28] from three observers were collected weekly prepartum and twice weekly postpartum. Milk production was measured for every milking and milk component analysis was performed for each cow weekly using a composite of samples from all three daily milkings. Composite milk samples were submitted to the Milk Laboratory Services of Dairy One Cooperative, Inc. Ithaca NY for milk component analysis. Daily feed intake was measured by weighing the offered and refused feed. Feed analysis was performed for prepartum diet and postpartum diet monthly through the Forage Laboratory Service of Dairy One Cooperative and dry matter intake (DMI) was calculated.

Serial energy balance was calculated according to the NRC (2001) equations [5, 14, 29]. Prepartum energy balance was calculated for the last three full weeks prior to calving using the following formula:

EB_{Pre} = Dietary Energy Intake – (Maintenance Requirement + Pregnancy Requirement)

Where:

EB_{Pre} = Prepartum energy balance (Mcal/d);

Dietary Energy Intake = average daily dry matter intake (kg) x dietary energy content (Mcal/kg dry matter);

Maintenance Requirement = metabolic body weight ($kg^{0.75}$) x 0.08 ($Mcal/kg^{0.75}$);

Pregnancy Requirement = (0.00318 x day of gestation – 0.0352) / 0.218.

Postpartum energy balance was calculated twice weekly for one and a half weeks starting at the first body weight measurement postpartum usually on day three postpartum. The formula used for postpartum energy balance was:

EB_{Post} = Dietary Energy Intake – (Maintenance Requirement + Lactational Requirement)

Where:

EB_{Post} = Postpartum energy balance (Mcal/d)

Maintenance Requirement = metabolic body weight ($kg^{0.75}$) x 0.08 ($Mcal/kg^{0.75}$);

Lactational Requirement = 0.0929 x milk fat percentage + 0.0563 x milk true protein percentage + 0.0395 x milk lactose percentage.

CNCPS model predictions on nutrient requirements

Feed chemical and kinetic composition and animal performance information was used to model metabolizable energy and metabolizable protein partitioning. The model consisted of two simulations per cow, one prepartum and one postpartum, using the Cornell Net Carbohydrate and Protein System (CNCPS) version 6.1 [30]. Based on feed composition from Cargill Inc. (feedstuffs provider) diet reports, a composite feed diet was created for pre and post partum cows using CNCPS, version 6.1 library. Maintenance requirements (MR) were adjusted to the expected changes during these physiological stages (i.e. pre and post partum) in organ mass, body weight and body condition, the level of activity as well as environment. Body weight and body condition score changes were calculated from predicted tissue energy losses and gains NRC (2001) and Fox et al (2004) [31]. Adjustments to MR for energy expended in daily physical activity was computed for the predicted amount of time standing, number of

position changes, and distance walked for tied-stall animals. Since this study was carried out in the period of winter-spring, heat stress was considered inconsequential and thus not affecting MR. The energy cost of excreting excess urea was calculated by subtracting it from ME intake [30]. In addition, the model predicted pregnancy, lactational, and mammary growth requirements according to the physiological stage. Balances on dietary ME and MP were reported in Mcal/d and g/d respectively and as the percent of requirements.

GTT and surrogate analysis

Individual prepartum and postpartum GTT results were analyzed by calculating the area under the curve (AUC) for glucose in mg/dl and insulin in μU/mL using the trapezoid rule. To account for possible differences in baseline glucose and insulin, the AUC over the baseline was calculated. Baseline for glucose and insulin was defined as the average concentration for the sample collected at -15 and -1 min. Glucose half life (T_{50}) was calculated using the formula: $T_{50} = \ln(2)/k$, where k = the elimination rate constant [32]. The elimination rate constant (k) was estimated by modeling the glucose clearance curve as an exponential decline model with non-zero asymptote using PROC NLMIXED of SAS version 9.2. The model used was: [glucose] = $(A_0 - A_{asym}) \times e^{-kt} + A_{asym}$, where A_0 = the extrapolated plasma [glucose] assuming instantaneous mixing at time = 0, t = time after infusion, A_{asym} = asymptotic plasma [glucose] when time » 0. In addition, insulin responsiveness in μU/mL (insulin peak – baseline insulin) and baseline glucose (mg/dl) to insulin (μU/mL) ratio calculated.

In addition to the GTT, additional surrogate insulin resistance tests were used to characterize changes of insulin resistance over time. Surrogate insulin sensitivity measures using steady-state conditions calculated were: Quantitative insulin-sensitivity check index (QUICKI) = $1/ (\log_{10}[\text{insulin}] + \log_{10}[\text{glucose}])$; Revised QUICKI (RQUICKI) = $1/(\log_{10}[\text{insulin}] + \log_{10}[\text{glucose}] + \log10[\text{NEFA}])$; Homeostatic Model

Assessment (HOMA) = ([glucose] + [insulin])/22.5; glucose to insulin ratio = [glucose]/[insulin]; and insulin sensitivity = 1/[insulin]. All [glucose] was in mg/dl except in the HOMA formula where [glucose] was in mMol/l, all [insulin] was in μU/mL and [NEFA] was in mMol/L

LH pulse frequency analysis

Identification of LH pulses and pulse characteristics analysis were performed with the PULSAR algorithm [33] using PC PULSAR version 1.3A (PC-Pulsar, Gitzen and Ramirez, University of Illinois). Cows experiencing LH surge during the LH pulse sampling window, which was defined as a continuous plasma LH concentration of > three times the average concentration of all cows, was excluded from LH pulse analyses.

Statistical analyses

All data were presented as means ± SEM. Continuous outcomes were transformed as necessary to satisfy model assumptions. A repeated-measures ANOVA was performed using MIXED procedure in SAS version 9.2 with first-order autoregressive covariance structure to evaluate the difference in hormone and metabolite concentration, surrogate insulin sensitivity, NRC energy balance calculations, milk production, body-weight, DMI and follicle growth over time between groups,. To test the difference in GTT and CNCPS modeling outcomes between group and time (prepartum or postpartum GTT), a mixed model ANOVA was performed with the random variable of time nested within cow and the fixed effect variables group and time using JMP Pro version 9. Generalized linear model regression was used to determine the differences in follicular fluid analyses, LH pulse analysis results and Day of specific events between groups. A Poisson model was used for LH pulses per 6 h. Two-way interactions were tested for all applicable analyses. When applicable, Tukey's HSD post

hoc test was performed to determine differences between categories to account for multiple testing. For all analysis, $P < 0.05$ was used as the threshold for significance.

RESULTS

Follicle fate

There were 28 cows that were classified as ovulatory that reached circulating estradiol concentrations of ≥2.0 pg/ml, 12 cows that were classified as non-ovulatory that had reached a follicle diameter of ≥ 15mm and did not have circulating estradiol levels of 2.0 pg/ml, and 12 cows that were classified as non-ovulatory that had a follicle that failed to grow after reaching > 10 mm in diameter. There were no significant differences between these two subgroups of non-ovulatory cows in any of the analyses and therefore they were combined to a single non-ovulatory group consisting of 24 cows.

The average days postpartum at group assignment was Day 14.7 (range from 9-19) postpartum. There was a tendency ($P = 0.09$) for non-ovulatory cows to have later group assignment (Day 15.5 ±0.7) compared with ovulatory cows (Day 14.1 ± 0.4). All four control ovulatory cows ovulated by 76 hours after achieving circulating estradiol concentration of ≥ 2 pg/mL. There were five control non-ovulatory cows with two cows achieving follicle size of 15 mm in diameter and circulating estradiol remained < 2pg/mL one day after achieving that follicle size, and three cows had follicles that failed to grow after achieving follicle size of ≥ 10 mm. None of these five control non-ovulatory cows ovulated the first dominant follicle postpartum and only one of these cows ovulated on a subsequent follicular wave by 30 days postpartum. Circulating progesterone concentrations reached ≥ 1ηg/mL in all control ovulatory cows by five days after ultrasound determined ovulation whereas the control non-ovulatory cows did not have increased circulating progesterone by 30 days postpartum except in one control non-ovulatory that ovulated in a subsequent follicular wave.

A subset of cows that had the first dominant follicle aspirated was examined daily by ultrasound examinations to determine the fate of the subsequent follicular wave dominant follicle. Sixteen ovulatory cows were followed up and eleven cows (69%) ovulated the subsequent dominant follicle, four cows developed a follicular cyst which was defined as a dominant follicle reaching > 20 mm in diameter and persisting for > one week and one cow had the subsequent dominant follicle regress after reaching > 10 mm. Fifteen non-ovulatory cows were followed up of which six cows (40%) ovulated the subsequent follicle, three cows developed follicular cysts and six cows had the subsequent follicle regress after reaching > 10 mm in diameter. The average interval between follicle aspiration and ovulation of the next dominant follicle in cows that did ovulate was 10.4 (range = 5-15) days.

The size of the first dominant follicle postpartum was not different between ovulatory and control ovulatory, or control non-ovulatory and non-ovulatory cows. There was a tendency for non-ovulatory cows to have smaller follicles compared with ovulatory cows at the same point postpartum ($P = 0.09$). When normalized by days from group assignment decision, there was no difference ($P = 0.88$) in follicle growth between groups (FIG. 5.1). Circulating estradiol for determining group assignment was also evaluated with time normalized to days from group assignment. The interaction between time and group was significant ($P = 0.002$) with ovulatory cows having increasingly higher circulating estradiol concentration compared with non-ovulatory cows and with the difference peaking at the day after follicle fate prediction (FIG. 5.1).

Endocrine and metabolic profiles

Circulating estradiol prepartum increased with time then dropped to near undetectable levels shortly after calving (FIG. 5.2A). No difference was detected between groups ($P = 0.98$) prepartum but a tendency ($P = 0.07$) for ovulatory cows to have higher circulatory estradiol early postpartum (Day 1-5 postpartum) was found.

Prepartum circulating progesterone declined with time (FIG. 5.2B). As with circulating estradiol, circulating progesterone levels dropped to near undetectable levels shortly after calving. There was no difference in plasma progesterone observed between groups ($P = 0.85$).

The DMI was higher in ovulatory cows (FIG. 5.3A) compared with non-ovulatory cows ($P = 0.0012$). Average percent daily feed refused was similar ($P = 0.58$) between ovulatory and non-ovulatory cows as was the average body weight ($P = 0.20$) and body condition score ($P = 0.78$). Milk production was not different (FIG. 5.3B) between groups after controlling for parity ($P = 0.23$). Energy balance calculated using the NRC 2001 formula was 2.07 Mcal/d higher for ovulatory cows (FIG. 5.4) compared with non-ovulatory cows ($P = 0.04$). There was a tendency ($P = 0.07$) for higher plasma NEFA in non-ovulatory cows compared with ovulatory cows (FIG. 5.5A) but the plasma BHBA (FIG. 5.5B) was not different between groups ($P = 0.19$). Prepartum energy and protein intake, growth requirement, balance and percent of requirement were significantly lower than postpartum predictions by CNCPS. Energy and protein intake was higher in ovulatory cows for both prepartum and postpartum simulations and resulted in higher positive energy balance prepartum and lesser negative energy balance postpartum compared with non-ovulatory cows. Pregnancy and lactational energy and protein requirements were not different between groups. The CNCPS simulation results are summarized in (Table 5.1)

Plasma glucose (FIG. 5.6A) was higher in non-ovulatory cows compared with ovulatory cows ($P = 0.007$) while the plasma insulin ($P = 0.20$) and IGF-1 ($P = 0.51$) were not different between groups (FIG. 5.6B-C). Of the surrogate insulin sensitivity measures, only glucose to insulin ratio was significantly different between groups ($P = 0.01$) while Insulin sensitivity ($P = 0.20$), QUICKI ($P = 0.84$), RQUICKI ($P = 0.17$) and HOMA ($P = 0.97$) were not different between groups.

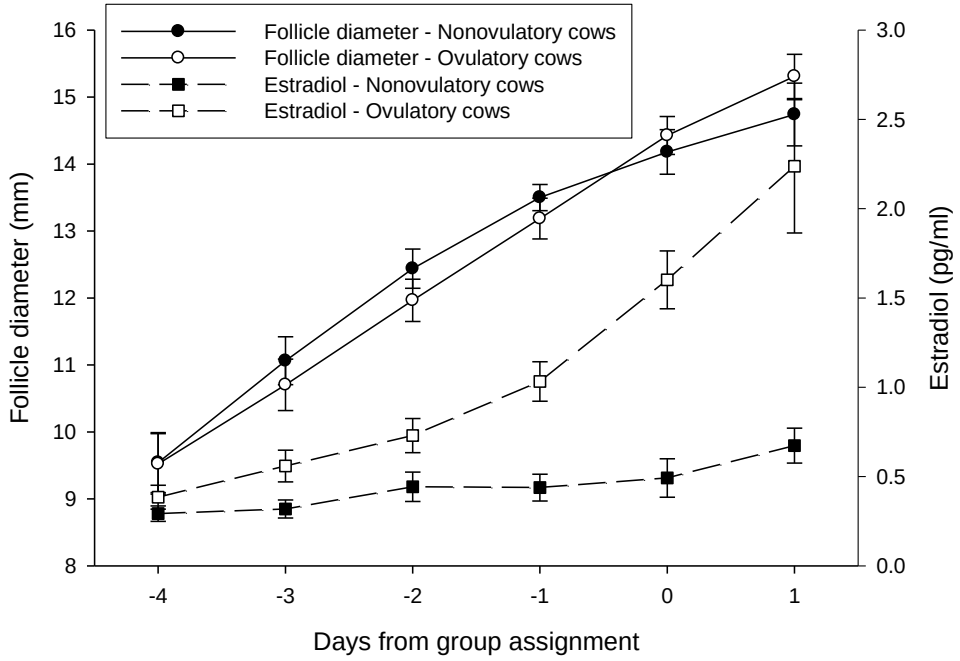
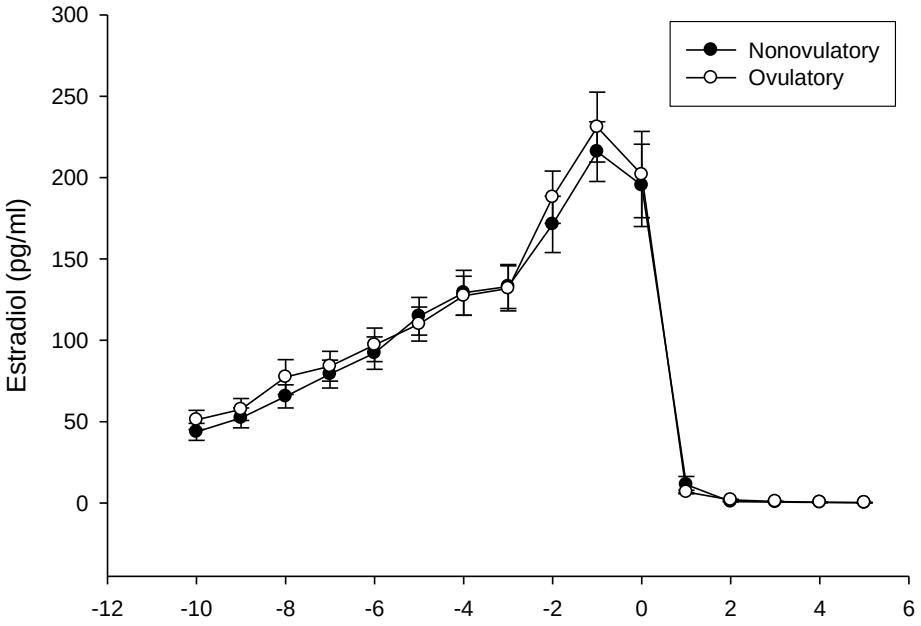


FIG. 5.1. Follicle growth characteristics were not different between ovulatory and non-ovulatory follicles after normalizing for the days from group assignment decision. Circulating estradiol was significantly higher in ovulatory cows compared with non-ovulatory cows from 3 days before group assignment.

A)



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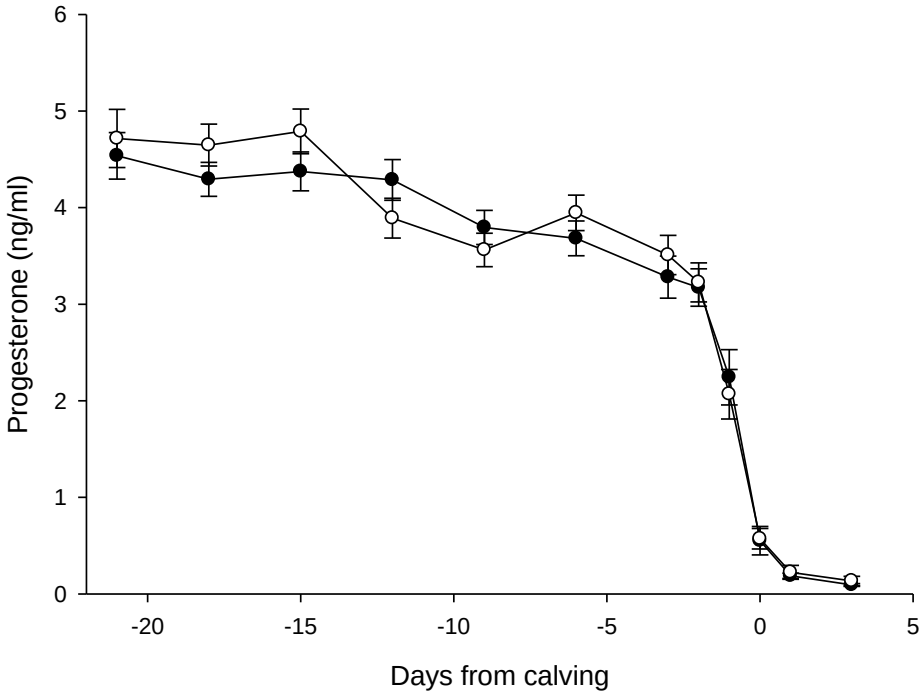


FIG. 5.2. Prepartum and early postpartum estradiol (A) and progesterone (B) concentrations were not different between ovulatory and non-ovulatory cows.

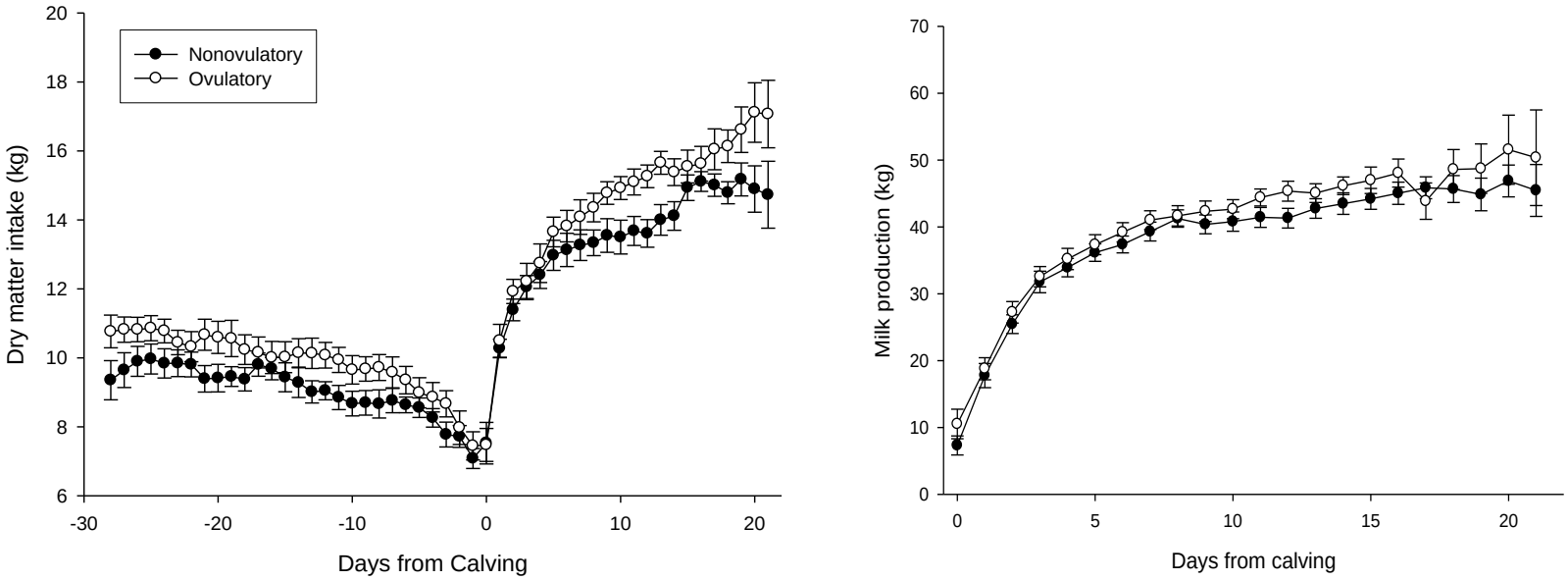


FIG. 5.3. Ovulatory cows had significantly higher (P = 0.0012) dry matter intake which was detected as early as 28 days before calving (A). Milk production was not significantly different (P = 0.23) between groups after accounting for parity effects (B).

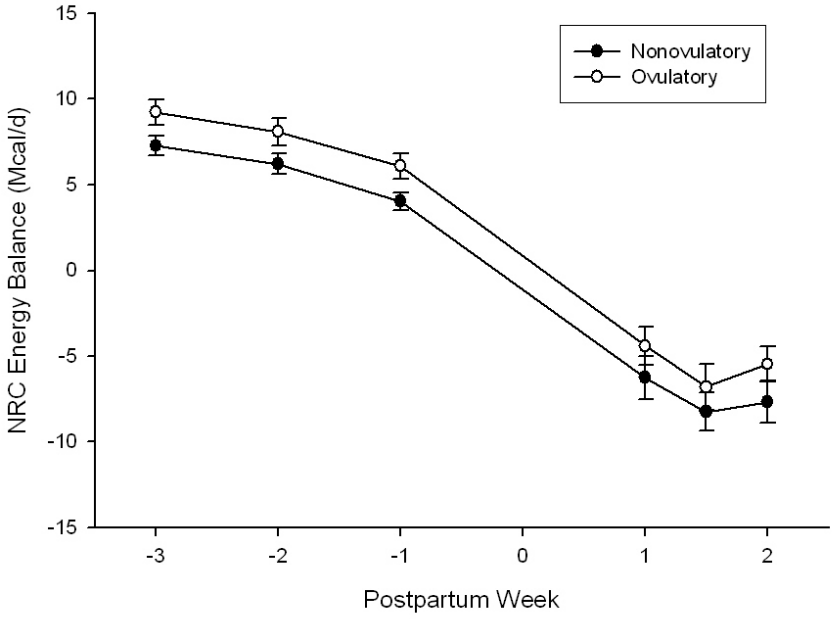
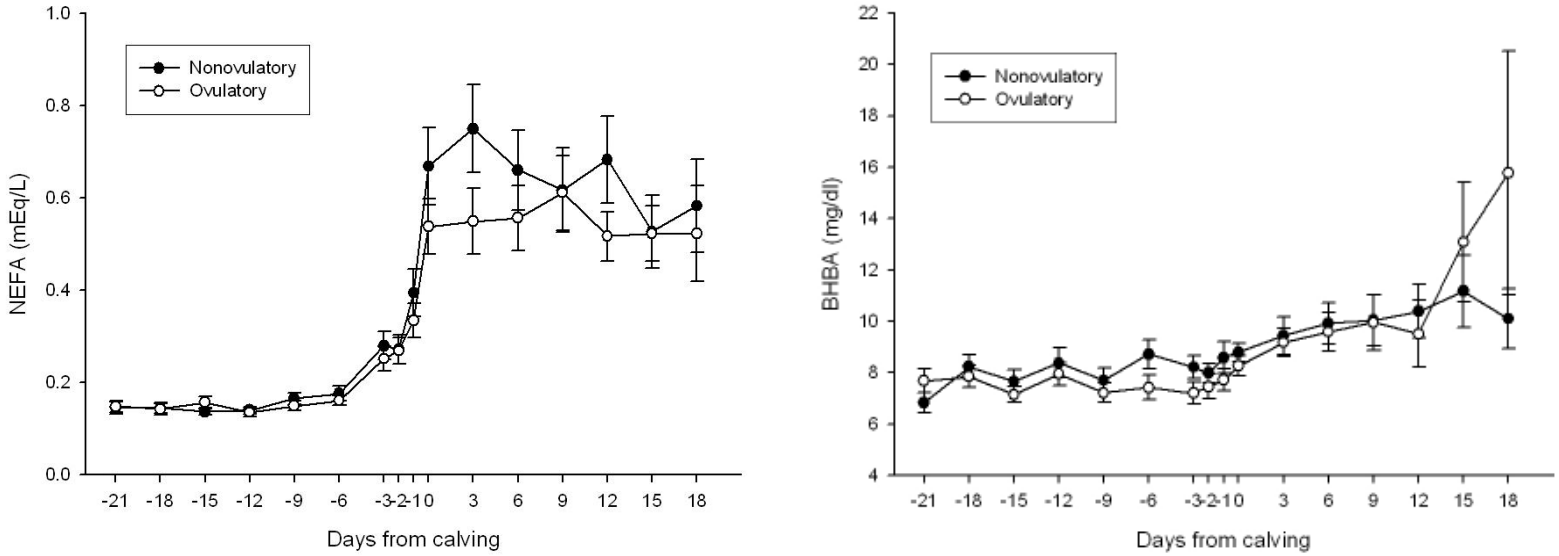


FIG 5.4. The energy balance was 2.07 Mcal/d lower in Non-ovulatory cows (P = 0.04) from the first simulation 3 weeks prepartum (C).



A) B)

FIG. 5.5. There was a tendency ($P = 0.07$) for higher NEFA in Non-ovulatory cows (A) while BHBA not different between groups (B).

GTT analysis

The average day-prepartum at the first GTT was 12.4 ±0.6 and was not different between groups ($P = 0.68$). Postpartum GTT results were associated with the prepartum GTT results but neither was correlated with circulating NEFA concentrations. Glucose AUC was significantly larger in prepartum GTT ($P < 0.001$) compared with the postpartum GTT and the glucose AUC was larger ($P = 0.01$) in postpartum non-ovulatory cows compared with postpartum ovulatory cows (FIG. 5.7). Baseline glucose was lower in prepartum GTT compared with postpartum GTT ($P < 0.001$) but was not different between groups. The rise in glucose after infusion was lower ($P = 0.008$) in the ovulatory postpartum GTT compared with postpartum non-ovulatory or prepartum GTT for either group. Glucose T_{50} was longer in prepartum GTT compared with postpartum GTT but were not different between groups. Insulin AUC was larger in prepartum GTT compared with postpartum GTT ($P < 0.0001$). There was an interaction between group and time for insulin AUC with non-ovulatory cows having numerically higher ($P = 0.13$) insulin AUC for prepartum GTT but a tendency ($P = 0.08$) for lower insulin AUC for postpartum GTT. Insulin responsiveness was also higher in prepartum GTT compared with postpartum GTT ($P < 0.0001$) and there was a significant interaction between group and time ($P = 0.03$). Insulin responsiveness was numerically higher ($P = 0.37$) in non-ovulatory cows for prepartum GTT but significantly lower ($P = 0.04$) for postpartum GTT compared with ovulatory cows. The GTT results are summarized in Table 5.2.

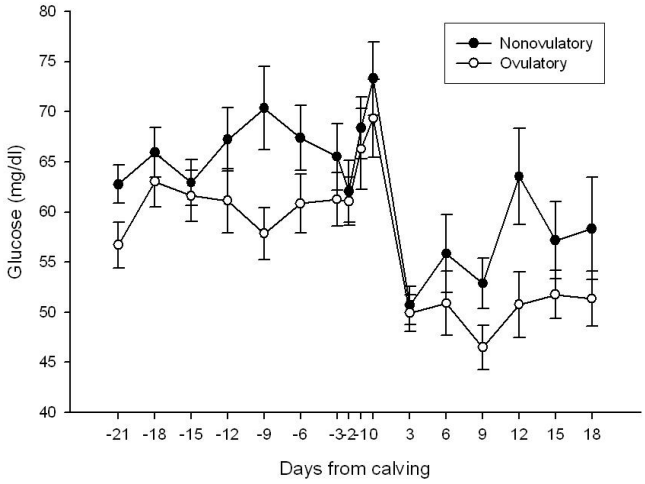
Table 5.1. Mean ± SEM for metabolizable energy (ME) and metabolizable protein (MP) as predicted by CNCPS simulations for a three week period prepartum and a one and a half week period postpartum.

		Prepartum		Postpartum	
		Non-ovulatory (n = 24)	Ovulatory (n = 28)	Non-ovulatory (n = 24)	Ovulatory (n = 28)
ME (Mcal/d)	Intake	35.34 ± 1.11[a]	39.73 ± 1.15[b]	51.99 ± 1.36[c]	59.58 ± 1.36[d]
	Maintenance	17.09 ± 0.37	17.82 ± 0.33	17.40 ± 0.37	17.74 ±0.30
	Pregnancy	6.15 ± 0.13	5.95 ± 0.13	NA	NA
	Lactation	NA	NA	50.07 ± 1.91	51.25 ±1.80
	Growth	2.80 ± 0.006[a]	2.82 ± 0.007[a]	2.50 ± 0.004[b]	2.53 ± 0.008[b]
	Balance	9.30 ± 0.90[a]	13.14 ± 1.00[b]	-18.00 ± 1.77[c]	-11.96 ± 1.47[d]
	% requirements	135.54 ± 3.19[a]	149.25 ± 3.65[b]	75.00 ± 1.79[c]	84.29 ± 1.95[d]
MP (g/d)	Intake	1,567.33 ± 54.04[a]	1,779.29 ± 55.94[b]	2,254.96 ± 63.80[c]	2,618.00 ± 64.86[d]
	Maintenance	665.92 ± 20.07[a]	746.96 ± 21.28[b]	790.42 ± 20.11[b]	905.18 ± 20.53[c]
	Pregnancy	369.83 ± 7.69	357.90 ± 8.02	NA	NA
	Lactation	NA	NA	2,054.94 ± 68.30	2, 108.33 ± 58.45
	Growth	271.48 ± 2.24[a]	275.53 ± 0.67[ab]	275.88 ± 0.57[b]	276.70 ± 0.00[b]
	Balance	260.05 ± 33.23[a]	398.90 ± 35.37[b]	-866.30 ± 52.16[c]	-672.11 ± 46.14[d]
	% requirements	119.17 ± 2.24[a]	128.14 ± 2.13[b]	72.50 ± 1.31[c]	79.71 ± 1.24[d]

[abcd] Within row, different superscripts are significantly different ($P < 0.05$) between simulation and/or group.

*NA not applicable for stage as prepartum cows are not lactating and lactating cows are not pregnant and have no requirements in those categories.

A)



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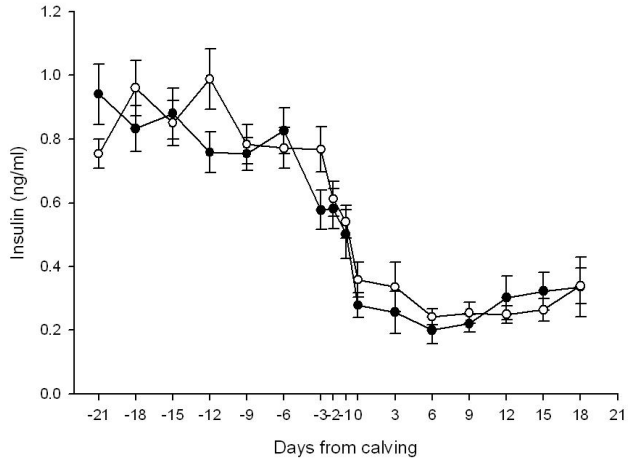

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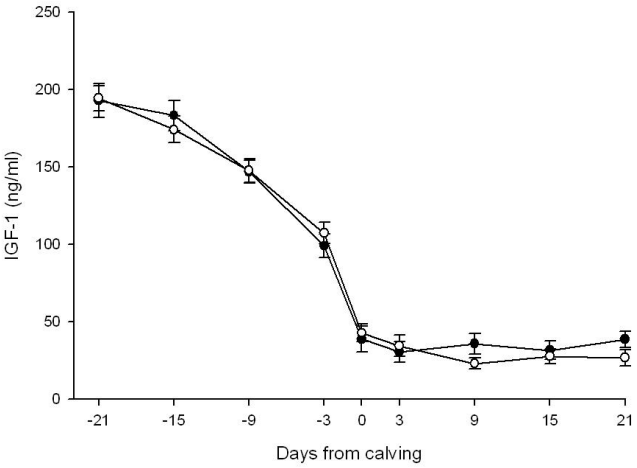


FIG. 5.6. Glucose was significantly higher (P = 0.007) in Non-ovulatory cows by 21 days prepartum and remained higher until 18 days postpartum (A). Insulin and IGF-1 was not different between groups (B, C).

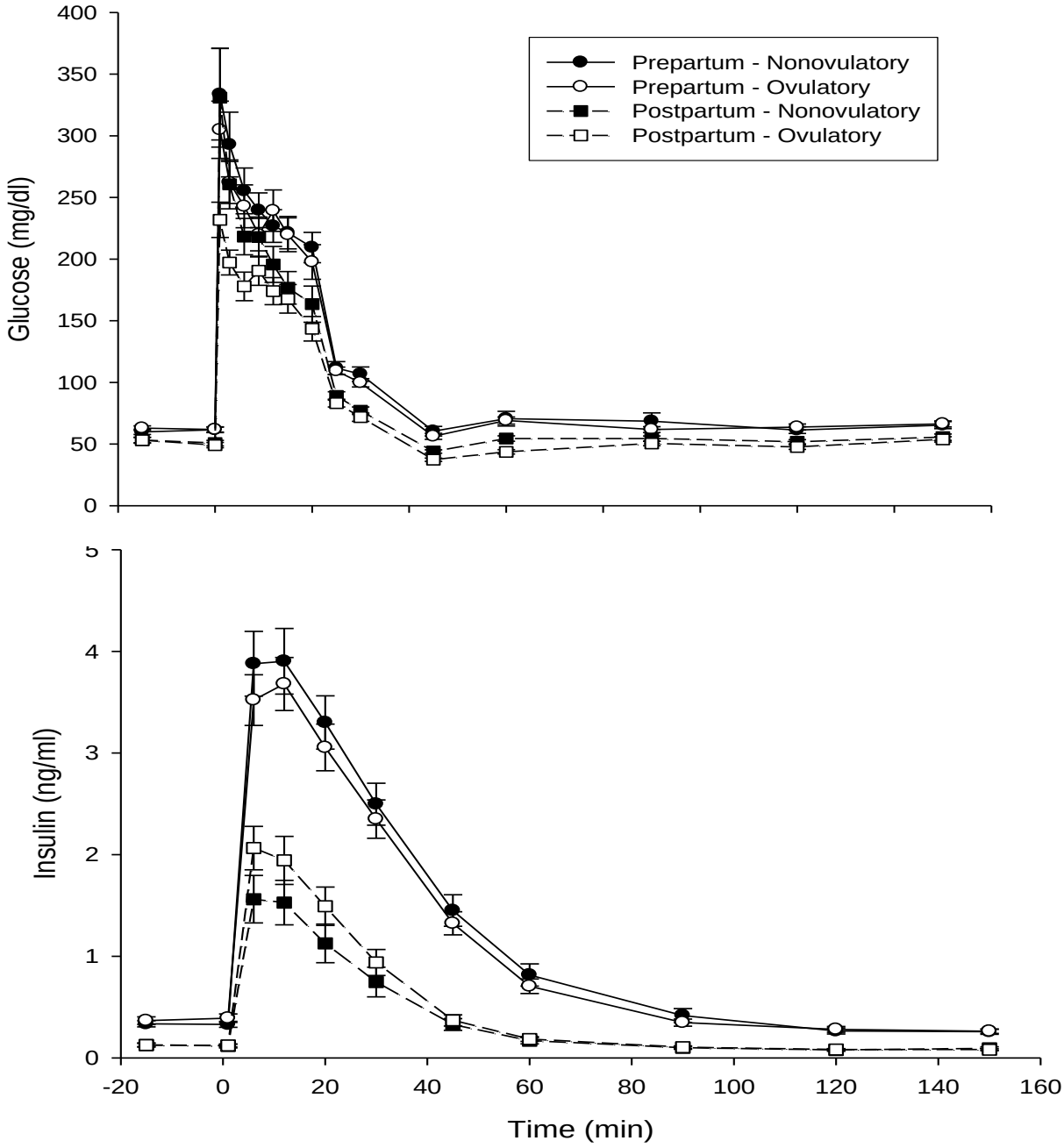


FIG. 5.7. Prepartum glucose tolerance test (GTT) had higher glucose and insulin baseline, rise and AUC compared with postpartum GTT ($P < 0.001$). Prepartum GTT results were similar between groups but in the postpartum GTT, ovulatory cows had lower glucose AUC ($P < 0.001$) and tended to have higher insulin AUC ($P = 0.08$) compared with non-ovulatory cows.

LH pulse frequency test

There were three cows that were obviously experiencing the LH surge, all in the ovulatory group, and these were excluded. The LH pulses per 6 h was higher (P = 0.002) in ovulatory cows (5.40 ± 0.35 peaks) compared with non-ovulatory cows (4.05 ± 0.27 peaks) and thus the inter-peak interval was also lower ($P < 0.001$). The amplitude of the LH pulses were not different between groups ($P = 0.73$) but the peak length was longer in non-ovulatory cows compared with ovulatory cows ($P = 0.005$). The results of LH pulse frequency test are summarized in Table 5.3.

Follicular fluid analysis

Exposure to LH surge induces luteinization of the follicular cells, resulting in a shift from estradiol to progesterone production. There were three cows, all in the ovulatory group that were experiencing the LH surge at the time of follicular fluid aspiration. These cows were considered a separate group for follicular fluid hormone analyses. Follicular fluid estradiol was significantly different ($P < 0.0001$) between groups with ovulatory (1630.78 ±106.51 ηg/mL) follicles having higher estradiol concentrations compared with non-ovulatory (566.99 ±115.53 ηg/mL) follicles while ovulatory with LH surge (1091.33 ±275.03 ηg/mL) follicular fluid estradiol concentration was not different from ovulatory or non-ovulatory follicles (FIG. 5.8A). Androsteinedione concentration was also significantly different between groups (P = 0.002) with ovulatory (8734.61 ±1434.27 ηg/mL) follicles having significantly higher androsteinedione concentrations compared with non-ovulatory (3675.80 ±924.86 ηg/mL) and ovulatory with LH surge (2158.80 ±898.55 ηg/mL) follicles (FIG. 5.8B). Progesterone concentrations in the follicular fluid was significantly ($P = 0.014$) different between groups with ovulatory with LH surge (146.53 ±21.32 ηg/mL) having significantly higher follicular fluid progesterone compared with ovulatory (75.56 ±8.26 ηg/mL) and non-ovulatory (86.39 ±8.70 ηg/mL) follicles (FIG. 5.8C). Follicular fluid

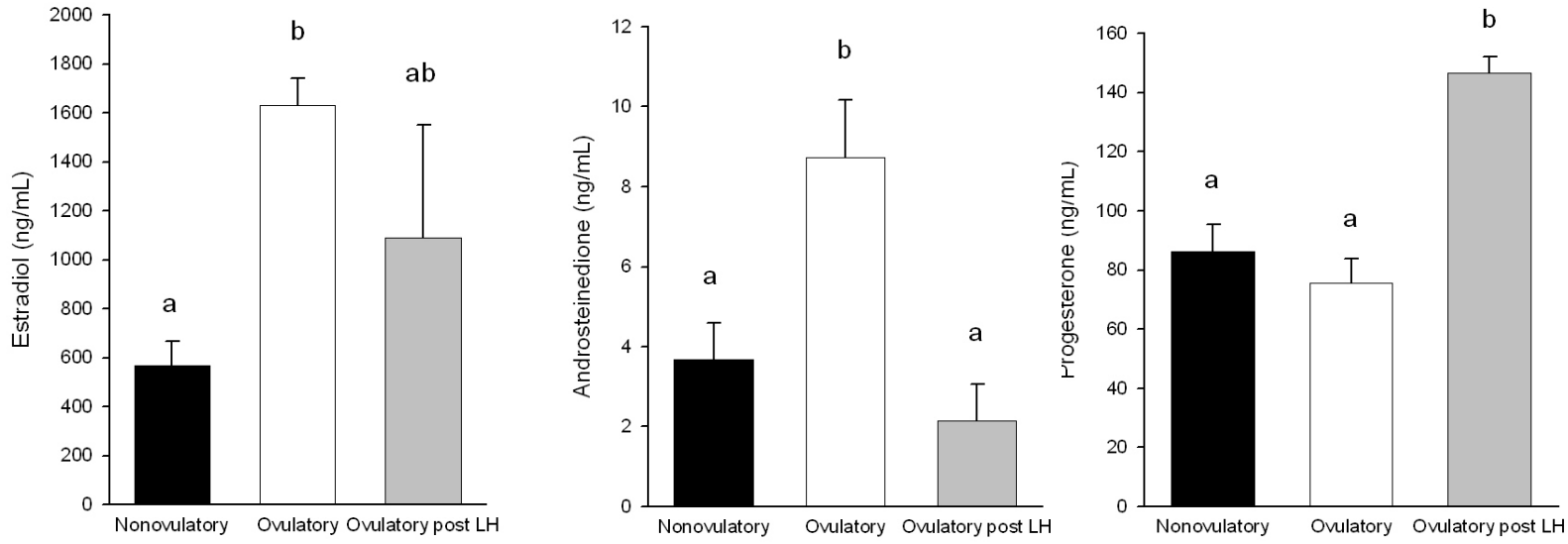
Table 5.2. Glucose tolerance test (GTT) results (Mean ± SEM) for prepartum and postpartum GTT by group.

	Prepartum GTT		Postpartum GTT	
	Non-ovulatory (N=24)	Ovulatory (N=28)	Non-ovulatory (N=19)	Ovulatory (N=24)
Glucose AUC (mg.min/dl)	5361.54 ± 505.40[a]	4575.86 ± 435.25[ab]	3917.84 ± 406.21[b]	2633.92 ± 238.72[c]
Insulin AUC (ng.min/mL)	124.12 ± 11.00[a]	105.10 ± 7.78[a]	37.32 ± 6.63[b]	48.47 ± 6.64[b]
Glucose baseline (mg/dl)	60.77 ± 1.79[a]	62.31 ± 1.71[a]	51.98 ± 1.63[b]	51.13 ± 1.99[b]
Insulin baseline (ηg/mL)	8.37 ± 0.75[a]	9.14 ± 0.93[a]	3.17 ± 0.51[b]	3.16 ± 0.39[b]
Glucose rise (mg/dl)	230.21 ± 35.64[a]	278.43 ± 20.46[a]	294.9 ± 38.49[a]	195.42 ± 14.15[b]
Insulin responsiveness (ηg/mL)	94.16 ± 7.60[a]	84.20 ± 5.92[a]	38.02 ± 5.33[b]	49.51 ± 5.44[c]
Glucose T_{50} (s)	17.12 ± 1.08[a]	15.47 ± 0.74[a]	12.95 ± 0.31[b]	12.10 ± 0.34[b]

[abc] Within row, different superscripts are significantly different ($P < 0.05$) between prepartum and postpartum, and/or group.

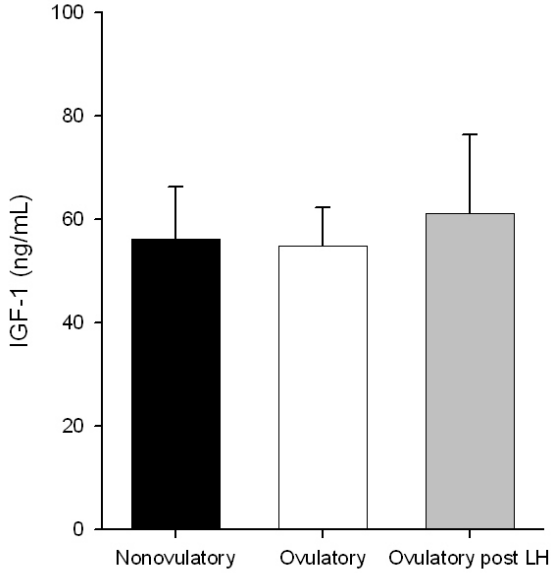
Table 5.3. Mean ± SEM for LH pulse frequency test.

	Non-ovulatory (N=19)	Ovulatory (N=20)	*P*
LH pulses/ 6 h	4.05 ± 0.27	5.40 ± 0.35	0.002
LH concentration (ηg/mL)	0.78 ± 0.01	0.91 ± 0.02	< 0.0001
Amplitude (ηg/mL)	0.61 ± 0.05	0.62 ± 0.08	0.73
Peak length (min)	53.7 ± 3.7	39.61 ± 2.96	0.005
Interpeak interval (min)	105.91 ± 7.54	70.58 ± 4.61	0.0002

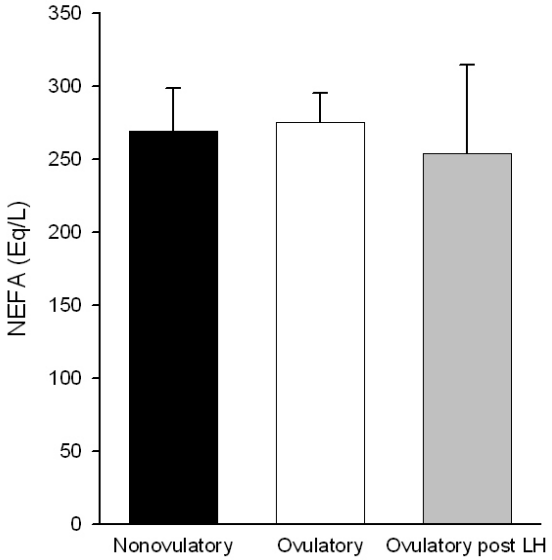


A) B) C)

FIG. 5.8. Follicular fluid profile for defective non-ovulatory, ovulatory and ovulatory with the LH surge) shortly before follicle aspiration. Estradiol (A) and androsteinedione (B) were higher in ovulatory follicles compared with non-ovulatory follicles while ovulatory with LH surge follicles had higher progesterone (C) compared with ovulatory and non-ovulatory follicles. Within panel, different superscript letters above bars indicate significant ($P < 0.05$) difference in means using Tukey's HSD multiple comparisons tests with 3 follicle groups.



A)



B)

FIG. 5.9. Follicular fluid total IGF-1 (A) and NEFA (B) were not different between groups. Within panel, different superscript letters above bars indicate significant ($P < 0.05$) difference in means using Tukey's HSD multiple comparisons tests with 3 follicle groups.

IGF-1 (P = 0.96) and NEFA (P = 0.84) were not significantly different between groups (FIG. 5.9A-B).

DISCUSSION

The group assignment to ovulatory and non-ovulatory was accurate for the nine control cows that were not aspirated. Previous studies have shown that there are cows that will fulfill the criteria for ovulation prediction but do not ovulate [5, 7, 25]. However, the cows that do not show a rise in circulatory estradiol do not ovulate [5]. The ability to produce estradiol does not guarantee the ability to ovulate. Approximately 60% of cows have first-dominant follicles postpartum that produce a rise in circulatory estradiol but only about 40% of cows ovulate [5, 7, 34]. In the present study, 54% of cows were assigned to the ovulatory group. Some of these follicles may not have gone on to ovulate. Some of the ovulatory cows that fail to ovulate the subsequent dominant follicle after aspiration (31% of ovulatory cows) may fall into this category. About 22% of cows ovulate the second follicular wave [34] and the non-ovulatory cows that ovulated the subsequent dominant follicle after aspiration may represent the second follicular wave ovulating cows.

This is the first description of the follicular fluid steroidogenic profile of the early postpartum bovine follicle that fails to produce the circulating estradiol rise typical of the dominant follicle. As expected; cows with high circulating estradiol, which were the ovulatory cows, had higher follicular fluid estradiol concentrations compared with non-ovulatory cows. The follicular fluid estradiol concentration for ovulatory cows was similar to reported values for preovulatory follicles [35, 36]. Follicular fluid androsteinedione was higher in ovulatory cows compared with non-ovulatory cows which show that the theca cell functions are impaired in non-ovulatory cows. Theca cells require LH stimulation to produce androsteinedione. Cows in the non-ovulatory group also have lower LH pulse frequency compared with ovulatory cows which shows

at least some degree of hypothalamic-pituitary-gonadal axis functions to be impaired in these cows. The follicular fluid androsteinedione concentrations were lower than reported for heifers or lactating cows [35, 36]. Progesterone was not different in the follicular fluid of ovulatory and non-ovulatory cows. Despite the reduced LH pulse frequency in non-ovulatory cows, the follicles were able to achieve ovulatory sizes albeit with a tendency for a one day delay in follicular growth compared ovulatory cows.

Prepartum GTT results were similar between groups for glucose and insulin responses. Pregnancy induces an insulin resistant state [37] and the prepartum GTT results were significantly different from the postpartum GTT responses [38]. Individual cow prepartum GTT results were associated with their respective postpartum GTT results as reported by others [17]. Non-ovulatory cows had significantly higher postpartum GTT glucose AUC, glucose rise and lower insulin responsiveness compared with ovulatory cows. There was also a tendency for postpartum GTT insulin AUC to be lower in non-ovulatory cows. Taken together, non-ovulatory cows had lower insulin response to the glucose infusion postpartum which resulted in impaired clearance of infused glucose compared with ovulatory cows indicating glucose tolerance in the postpartum non-ovulatory cows. Insulin resistance surrogates were evaluated and only glucose to insulin ratio was significantly different between groups. Surrogate tests utilize a ratio of glucose, insulin and NEFA in an assumed fasting steady state to identify individuals with insulin resistance without having to perform dynamic tests which are difficult to perform on a large scale. However these were not found to be more predictive of group than glucose levels. Plasma glucose was the only metabolite to be significantly different between groups with non-ovulatory cows having higher glucose concentration by 21 days prior to calving. This difference in circulating glucose was observed despite similar circulating insulin concentrations between groups also indicating glucose tolerance. The postpartum GTT insulin response to glucose challenge was lower in non-ovulatory cows which agree with the steady state observation.

However, the prepartum GTT insulin response was not different between groups yet the steady state glucose was different. The expected profile of high prepartum insulin and IGF-1 prepartum declining in the last two weeks of gestation [39] was seen in this study. However, the reported higher circulating IGF-1 postpartum in cows that ovulated the first dominant follicle as previously reported [5, 40] was not observed. Follicular steroidogenesis by the granulosa and theca cells requires insulin to respond appropriately to gonadotropin stimulation [22, 41, 42]. Insulin levels are low during the early postpartum period and supplementation does not increase LH pulse frequency directly [25]. Instead, insulin and IGF-1 affect the follicle directly by increasing the LH receptor numbers of the granulosa and theca cells, increasing the steroidogenic response to gonadotropin stimulation [42-45]. In the present study, LH pulse frequency was reduced and insulin levels not different between groups which suggest the steroidogenic defect is attributable, at least in part by the reduced gonadotropin.

The predicted ME and MP balance of non-ovulatory cows was lower compared with ovulatory cows. The ME and MP supply from feed intake was lower in non-ovulatory cows but the requirements for maintenance, growth, pregnancy and lactation was not different between group which indicates the difference in intake is the primary driver for the lower energy status in non-ovulatory cows. All cows were individually fed with no competition for food or limit to the amount of feed that can be offered, yet non-ovulatory cows had lower feed intake compared with ovulatory cows suggesting some appetite suppression in these cows. Negative energy balance decrease GnRH pulse frequency and result in a parallel decrease in LH pulse frequency [7, 14] which may be due to the increased sensitivity of the hypothalamus to the negative feedback of estradiol on GnRH release. There was a significant ($P = 0.02$) association between postpartum energy balance and LH pulse frequency in the present study (data not shown). LH pulse frequency increases after the nadir of energy balance which usually occurs on average by 12 days postpartum [14]. Despite the difference in ME and MP

balance; circulating BHBA and NEFA, which are predictors of negative energy balance, was not significantly different between groups although there was a tendency for higher NEFA in non-ovulatory cows. While higher levels of NEFA have been shown to impair oocyte maturation [46] and viability of granulosa and theca cells [20, 47], the NEFA concentration in the follicular fluid was not different between groups.

In conclusion, the follicles with and without steroidogenic defects can be identified prior to ovulation or follicle wave turnover using follicle growth patterns and circulating estradiol concentrations. The steroidogenic functions of theca cells from these follicles that do not produce rising circulatory estradiol are compromised at least partially by the decreased LH pulse frequency. Postpartum GTT shows impaired glucose clearance with a tendency for decreased insulin response in the steroidogenic defect cows although circulating insulin levels were not different between groups. Negative energy balance was more severe in cows with steroidogenic defect due to lower feed intake despite free access to food. The prediction method will allow further studies to determine if there are intrinsic defects of the follicle itself.

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CHAPTER 6

UTERINE HEALTH INFLUENCES FOLLICULAR FUNCTION IN POSTPARTUM DAIRY COWS.

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ABSTRACT

The objective of this study was to determine the association between uterine bacteria, uterine inflammation, systemic inflammatory response and the growth and ovulation of the first dominant follicle postpartum. Follicle fate of the first dominant follicle postpartum was predicted in 56 multiparous cows by using a combination of follicle growth characteristics and circulating estradiol concentrations. Follicular fluid was aspirated the day after follicle fate prediction in a subset of cows for endotoxin and paraoxonase evaluation. Uterine bacteria and inflammation was evaluated on 3 uterine samples from each cow collected at the day of calving, the day after follicle aspiration, and at 35 days postpartum. None of the cultured bacteria isolates were significantly associated with follicle fate individually or grouped by known uterine pathogens or potential uterine pathogen bacterial species. The presence of *Escherichia coli* (Estimate = -1.62 mm, 95% C.I. -2.93– -0.31) or *Pasteurella spp*. (Estimate = -4.02 mm, 95% C.I. -7.89– -0.15) in uterine samples at the day of calving was associated with slower follicle growth rate as was the presence of *Streptococcus uberis* (Estimate = -2.64 mm, 95% C.I. -5.07- -0.21) or *Mycoplasma spp*. (Estimate = -2.19 mm, 95% C.I. -10.13- -2.19) in the uterine sample collected the day after follicle aspiration. There was a significant association between follicular fluid endotoxin and follicular fate (OR for non-ovulatory = 4.99 per EU/mL endotoxin, 95% C.I. 2.87 – 13.96). Haptoglobin levels were higher in non-ovulatory cows at the day of calving (Estimate = 0.46 ± 0.12) and on at day 3 after calving (Estimate = 0.39, ± 0.12). Paraoxonase activity in follicular fluid was significantly associated with the paraoxonase activity in plasma however; plasma paraoxonase was not different between non-ovulatory and ovulatory cows.

INTRODUCTION

At parturition, most cows have some bacterial contamination of the uterus which is cleared by the immune system in healthy cows (Williams et al., 2005; Sheldon et al., 2008). Bacterial contamination of the uterus postpartum is associated with both uterine disease and impaired ovarian function (Opsomer et al., 2000; Sheldon and Dobson, 2004; Williams et al., 2007). Cows with more bacterial contamination postpartum have slower follicular growth and decreased estradiol production (Sheldon et al., 2002; Williams et al., 2007). Most cows will develop a dominant follicle in the first follicular wave postpartum (Crowe, 2008). The difference between follicles that will go on to ovulate and those that do not is the rise in circulating estradiol concentrations (Beam and Butler, 1997; Butler et al., 2006). Non-ovulatory follicles appear normal by ultrasound examination; however, most do not produce estradiol that is detectable in circulation and have slower follicle growth rate. This pattern is similar to the effects observed in cows with heavy bacterial contamination.

The presence of bacteria induces an inflammatory response in the uterus including the increase of neutrophils in uterine samples resulting in endometritis; and release of inflammatory products such as interleukins, tumor necrosis factor-α (TNFα), prostaglandins, reactive oxidative species and nitric oxide (Gilbert, 2011). Endometritis has been associated with impaired reproductive outcomes (Gilbert et al., 2005; Barlund et al., 2008; Cheong et al., 2011) and anovulatory conditions (Galvao et al., 2010). Bacterial products such as endotoxin can affect the uterine (Herath et al., 2009) and ovarian functions (Herath et al., 2007; Williams et al., 2008) directly but also has indirect effects by impairing gonadotropin release (Peter et al., 1990b; Battaglia et al., 1997; Crowe, 2008). Inflammatory products such as TNFα can also directly impair steroid production (Williams et al., 2008) and induce release of

positive acute phase proteins such as haptoglobin. Haptoglobin has been shown to have strong association with uterine diseases in dairy cows, making it a useful marker of systemic response to the uterine infection (Huzzey et al., 2009; Chan et al., 2010). Conversely, endotoxin and inflammatory products decrease synthesis of negative acute phase proteins such as paraoxonase (Feingold et al., 1998). Paraoxonase is produced by the liver and has protective effects on oxidative stress and lipid metabolism (Antoncic-Svetina et al., 2011).

The objective of this study was to determine the association between uterine bacteria, uterine inflammation, systemic inflammatory response and the growth and ovulation of the first dominant follicle postpartum.

MATERIALS AND METHODS

Animals

Holstein cows from the Cornell Teaching and Research Dairy Unit were used in this study. A total of 53 multiparous cows were enrolled for the study 28 days before the due date until 35 days in milk (DIM). Cows were housed in tie-stalls with free access to water and individually fed a total-mixed ration formulated to meet or exceed the NRC 2001 recommendations. Rectal temperature was measured daily starting from 10 days before the due date and fever was defined as a rectal temperature of $\geq$ 39.5°C. Acute puerperal metritis was defined as fever with reddish-brown foul smelling vaginal discharge. All procedures were approved by the Cornell University Institutional Animal Care and Use Committee.

Follicle function evaluation

The first dominant follicle postpartum was evaluated for follicle growth characteristics and estradiol production. Ovarian structures were measured daily by

transrectal ultrasonography using a 7.5 MHz linear probe (Aloka Inc. Wallingford, CT) starting 7 DIM. Follicle sizes were measured using internal calipers and the location mapped for all follicles ≥ 5 mm. The first follicle to reach 10 mm in diameter was considered the first-dominant follicle postpartum. Circulating estradiol concentrations were measured daily. Cows were considered ovulatory (OV) if circulating estradiol was ≥ 2 pg/mL in the presence of a follicle > 10mm in diameter and the follicle was considered non-ovulatory (NOV) if the first-dominant follicle postpartum failed to grow or if circulating estradiol did not reach ≥ 2pg/mL the day after the follicle reached a diameter of 15 mm. The first dominant follicle postpartum was aspirated by ultrasound guided fine-needle aspiration the day after reaching at least one of the following criteria: 1) follicle reached a diameter of > 15 mm, 2) follicle failed to grow after reaching a diameter of > 10 mm, or 3) circulating estradiol concentrations reached ≥ 2 pg/mL.

Uterine sampling

Uterine samples were collected three times for each cow using low-volume uterine lavage (Gilbert et al., 2005). The first sample was collected on the day of calving, the second was collected the day after follicle aspiration, and the third at 35 DIM. The perineum was cleansed and a sterile pipette introduced through the cervix into the uterus. Sterile saline (20mL) was infused into the uterus and the uterus was massaged per rectum before recovery. A sterile swab was soaked in the uterine sample for 5 minutes then put into transport media (BBL™ Port-A-Cul™ Tube, Becton Dickson and Co., Sparks MD) to be submitted to the Animal Health Diagnostic Center at Cornell University for aerobic and anaerobic bacteria, mycoplasma and ureaplasma culture. Anaerobic bacterial culture was not performed when samples could not be submitted to the laboratory promptly which was during

holidays and weekends.

Aerobic cultures were performed by first inoculating samples onto Columbia agar with 5% sheep blood, chocolate agar, eosin methylene blue agar, and colistin/nalidixic acid agar plates. After 24 to 48 hours of growth, bacterial colonies of varying morphologies were tested using manual biochemical tests. Complete identification was done using the Sensititre automated bacterial identification system (TREK Diagnostic Systems Inc., Cleveland, OH), used according to the CLSI guidelines. Semi-quantitative bacterial concentration data was collected for each aerobic bacteria isolate and the categories recorded were: Few, Moderate, and Many.

Anaerobic cultures were performed by inoculating samples onto Brucella agar, phenylethyl alchohol agar, Laked Blood with Kanamycin and Vancomycin agar, and Bacteroides Bile Esculin Agar plates. Bacterial isolates were confirmed as strict anaerobes by failure to grow in the presence of oxygen, and identified using a combination of staining and resistance characteristics.

Mycoplasma and ureaplasma were cultured from samples by inoculation onto agar media specific for the growth of these organisms and identified visually using a dissecting microscope. For ureaplasma, samples were also inoculated into ureaplasma broth and were subcultured if the appropriate color change was observed.

All *Escherichia coli* isolates were saved for phylogenetic typing. Phylogenetic group (A, B1, B2 or D) was determined for the *Escherichia coli* isolates using polymerase chain reaction as previously described (Clermont et al., 2000).

Isolated bacteria were classified as known uterine pathogens: *Trueparella pyogenes* (formerly known as *Arcanobacterium pyogenes*), *Escherichia coli*, *Fusobacterium necrophorum*, *Fusobacterium nucleatum*; potential uterine pathogens: *Histophilus somni*, *Mannheimia haemolytica*, *Pasteurella multocida*, *Peptostreptococcus spp*., *Streptococcus uberis*, *Staphylococcus aureus*; and bacteria

not recognized as uterine pathogens (Sheldon et al., 2002). Mycoplasma and ureaplasma were tested individually.

Uterine lavage samples were tested using a urinary reagent strip Multistix 10 SG (Bayer Corp., Elkart, IN) immediately after collection by placing a drop of recovered fluid directly on each reagent strip. Leukocyte esterase, protein and pH values were recorded for analysis. Cytology was also performed on the uterine samples as previously described (Cheong et al., 2012). Cytology slides were prepared using a cytocentrifuge and stained (Camco Stain Pak, Cambridge Diagnostic Products Inc., Fort Lauderdale, FL). 200 cells were counted excluding erythrocytes and were characterized as polymorphonuclear leukocytes (PMNL) cells (which were mostly neutrophils), uterine epithelial cells, lymphocytes and macrophages.

Hormone and inflammatory markers

Circulating estradiol concentrations were determined daily starting day 7 postpartum using RIA (Serono-Maia, Cortland Manor, NY) as previously described (Butler et al., 2004) except the extraction step was performed using 3 mL Benzene:Toulene (2:1 vol/vol) for 200 µL of plasma. Endotoxin concentrations in follicular fluid was determined using a commercial kit (Endpoint Chromogenic LAL Assay, Lonza Walkersville Inc, Walkersville, MD) as previously described (Herath et al., 2007; Williams et al., 2007). Follicular fluid samples were diluted 1:50 and heat inactivated at 75° C for 30 minutes. Each sample was serially diluted until the values were within the standard curve range. Each dilution was tested by spiking the samples with a known quantity of endotoxin. Recovery of > 80% was considered to have no inhibition of the reaction.

A subset of 20 cows was randomly chosen and used to determine the association between inflammatory markers and follicle fate (10 ovulatory and 10 non-

ovulatory cows). Plasma samples on day -21, -16, -9, -3, 0, 3, 9 and 15 from calving were analyzed for each cow. Plasma haptoglobin concentration was determined in plasma samples using a guaiacol test as previously described (Jones and Mould, 1984). Paraoxonase levels in plasma and follicular fluid were determined using a commercial kit (OXItek, ZeptoMetrix Corp. Buffalo, NY). Intra and inter assay coefficient was 1.2% and 16%; and 2.8% and 5% respectively for haptoglobin and paraoxonase.

Statistical analysis

Association between parity, calving-ease score, calf sex, acute puerperal metritis, and retained fetal membranes on the dichotomized follicle fate was evaluated using PROC LOGISTIC of SAS version 9.2 (SAS Institute Inc., Cary, NC). Two-way interactions were tested and all variables with $P < 0.20$ were offered in a multivariable model. The final model was built using a backwards stepwise method and variables retained if $P < 0.05$.

Uterine sampling results were tested individually. Continuous outcomes were dichotomized if the outcome was associated with follicle fate at $P < 0.20$. A receiver operating characteristic (ROC) curve was used to determine the optimal cutoff point in JMP pro 9 (SAS Institute Inc., Cary, NC). Association with follicle fate was performed using PROC LOGISTIC or SAS version 9.2. Bacterial association with follicle fate was tested for individual bacterial presence in individual uterine samples at each time point using PROC LOGISTIC of SAS version 9.2. Bacterial isolates with $P < 0.20$ was offered to build multinomial model for each uterine sample time point and isolates retained in the model if $P < 0.05$. Bacteria isolates were tested individually and also grouped by the presence of at least one known pathogenic bacteria species in any of the three uterine sample time points, and tested again

including potential pathogenic bacteria isolates using PROC LOGISTIC of SAS version 9.2.

The association between bacterial isolates and follicle growth and between plasma haptoglobin and paraoxonase on follicle fate were evaluated using repeated measures analysis (PROC MIXED of SAS version 9.2) with first order auto-regressive covariance structure. Two-way interactions were tested for all models and when significant, a post-hoc multiple comparison test using Bonferroni correction was performed. Distributions of continuous variables were tested and transformed as necessary to fit model assumption. Variables were considered significant if $P < 0.05$. Continuous variables estimates were reported with ± standard error.

RESULTS

Uterine health on ovulation

There was no association between parity, calving-ease score, calf-weight or interactions with follicle fate. There was a tendency for cows that had bull calves to be non-ovulatory (OR = 2.80, 95% C.I. 0.89 – 8.86; $P = 0.080$). Retained fetal membranes were not associated with follicle fate but cows that developed acute puerperal metritis tended to be non-ovulatory (OR = 3.60, 95% C.I. 0.94 – 13.78; $P = 0.062$) and the results are shown in **Table 6.1**. A multivariable model was tested but did not retain any variables with $P < 0.05$. The proportion of PMNL in the first uterine sample and the pH of the first uterine sample tended to be associated with follicle fate and the optimal cutoff point determine by ROC analysis was 35% PMNL and pH 8.5. Cows that had < 35% PMNL in the first uterine sample were more likely to be ovulatory (OR = 4.53, 95% C.I. 1.09 – 18.89; $P = 0.038$) and cows that had the first uterine sample with pH < 8.5 were more likely to be ovulatory (OR = 5.13, 95% C.I. 1.53 – 17.28; $P = 0.008$). All other reagent strip and cytological outcomes in any

Table 6.1 Distribution of calving related risk factors and uterine health condition and the *P*-value for the association with follicular fate using logistic regression.

		Ov (n = 28)	NOV (n= 25)	*P*
Parity				0.61
	2	14	15	
	3	8	7	
	4	6	3	
				0.70
Calving ease score	1	20	15	
	2	7	7	
	3	1	3	
Calf Sex				0.08
	Female	15	8	
	Male	13	17	
Calf weight (kg)				0.30
	Average	47.04	47.12	
	SEM	1.18	1.07	
Retained Fetal Membrane				0.77
	Yes	3	2	
	No	25	23	
Acute Puerperal Metritis				0.062
	Yes	4	9	
	No	24	16	

of the three uterine samples collected from each cow were not associated with follicle fate.

Bacteriology and ovarian function

The proportion of cows that had bacteria isolated from uterine cultures decreased from 50/53 (87%) to 41/53 (77%) to 27/53 (51%) at the day of calving, day after aspiration and at 35 DIM respectively. The list of bacteria isolated from uterine samples is given in **Table 6.2**. There were no significant associations between the presences of any individual bacteria in any of the 3 uterine sampling on follicle fate. Grouping bacteria by the presence of a known pathogen in any of the 3 uterine cultures, 73.1% of cows had pathogenic bacteria in at least one of the uterine cultures, and when potential pathogens were included, 80.8% of cows had known or potential pathogens isolated from uterine samples. The association between follicle fate and the presence of a known bacterial pathogen (OR = 1.83, 95% C.I. 0.53- 6.33; $P = 0.34$) or combined known and potential bacterial pathogen (OR = 0.50, 95% C.I. 0.12 – 2.04; $P = 0.33$) were not significant. All cows that had mycoplasma and cows that had ureaplasma isolated from uterine samples also had a known pathogenic bacterium isolated from at least one of the uterine samples.

The effect of parity was not significant in any of the models for follicle growth; however, time was significant and was included in all repeated measures models. First uterine sample bacteria isolates that had potential association with follicle growth at $P < 0.20$ and tested in the multivariable model were: Alpha-hemolytic streptococcus, *Trueperella pyogenes*, *Corynebacterium spp*., *Escherichia coli*, *Pasteurella spp*., *Streptococcus bovis*, and *Actinomyces spp*. In the final model for bacteria isolates in the first uterine sample, *Escherichia coli* and *Pasteurella spp*. were retained. Follicle growth was slower in cows that had *Escherichia coli* isolated

Table 6.2 Bacteria species isolated from uterine samples collected on the day of calving, the day after follicle aspiration and at 35 DIM.

Aerobic	**Anaerobic**
Actinobacillus spp.	*Actinomyces spp.*
Trueperella pyogenes (formerly *Archanobacterium pyogenes*)	*Actinomyces viscosus*
Bacillus spp.	*Bacteroides fragilis*
Corynebacterium spp.	*Bacteroides ovatus*
Escherichia coli pathotypes A, pathotypes B1, pathotypes B2, pathotypes D,	*Bacteroides vulgatus*
Escherichia fergusonii	*Clostridium spp.*
Enterococcus faecium	*Clostridium perfringens*
Gardnerella spp.	*Eggerthella lenta*
Histophilus somni	*Fusobacterium spp.*
Mannheimia haemolytica	*Fusobacterium necrophorum*
Moraxella osloensis	*Fasobacteroim nucleatum*
Pasteurella spp.	*Fusobacterium varium*
Pasteurella multocida	*Peptostreptococcus spp.*
Proteus mirabilis	*Peptostreptococcus asaccharolyticus*
Serratia plymuthica	*Peptostreptocuccus anaerobius*
Staphylococcus spp.	*Peptostreptococcus acnes*
Staphylococcus intermedius	*Prevotella spp.*
Coagulase-negative Staphylococcus	*Porphymonas*
Staphylococcus haemolyticus	
Streptococcus spp.	
Alpha-hemolytic Streptococcus	
Beta-hemolytic	
Group C	
Streptococcus dysgalactiae	
Group D, non-enterococcus	
Streptococcus bovis	
Non-hemolytic Streptococcus	
Streptococcus uberis	

from the first uterine sample (Estimate = -1.62 mm, 95% C.I. -2.93– -0.31; *P* = 0.017) as well as cows that had *Pasteurella spp*. (Estimate = -4.02 mm, 95% C.I. -7.89– -0.15; *P* = 0.042).

Second uterine sample bacteria isolates that had potential association with follicle growth and tested in the multivariable model were: Escherichia coli pathotype B1, Non-hemolytic streptococcus, *Pasteurella spp*., *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Peptostreptococcus spp*., *Prevotella spp*., *Bacteroides vulgatus*, and mycoplasma spp. In the final model for bacterial isolates in the second uterine sample retained *Streptococcus uberis*, mycoplasma spp. and *Escherichia coli* pathotype B1. Slower follicle growth occurred in cows that had *Streptococcus uberis* (Estimate = -2.64 mm, 95% C.I. -5.07- -0.21; *P* = 0.034) and mycoplasma spp. (Estimate = -2.19 mm, 95% C.I. -10.13- -2.19; *P* = 0.0041) but cows that had *Escherichia coli* B1 pathotype had faster follicle growth (Estimate = 2.67 mm, 95% C.I. 0.86 – 4.48; *P* = 0.0046).

Third uterine sample bacterial isolates that had potential association with follicle growth and tested in the multivariable model were: *Trueperella pyogenes*, *Bacteroides vulgatus*, *Pasteurella spp*., *Moraxella osloensis*, and Coagulase-negative Staphylococcus. The final model retained only *Moraxella osloensis*. Faster follicle growth was associated with the presence of *Moraxella osloensis* (Estimate = 4.57 mm, 95% C.I. 0.00 – 9.13; P = 0.05) isolated from the third uterine sample.

Endotoxin and inflammatory markers

Follicular fluid endotoxin levels were below the lowest standard in 26 of the 43 samples tested. There was a significant association between follicular fluid endotoxin and follicular fate (OR for non-ovulatory = 4.99 per EU/mL endotoxin, 95% C.I. 2.87 – 13.96; *P* = 0.038).

There was a significant interaction ($P = 0.017$) between days from calving and follicle fate on plasma haptoglobin (**Figure 6.1**). Within-day haptoglobin levels were higher in non-ovulatory cows at the day of calving (Estimate = 0.46 ± 0.12; $P < 0.0001$) and on at day 3 after calving (Estimate = 0.39, ± 0.12; $P = 0.0011$).

Paraoxonase activity in follicular fluid was significantly associated (Estimate = 1.06 ± 0.20; $P < 0.001$) with the paraoxonase activity in plasma (**Figure 6.2**). There were no significant interactions between days from calving and follicle fate on plasma paraoxonase activity. Plasma paraoxonase was not different (Estimate = 6.26 ± 9.14; $P = 0.50$) between non-ovulatory and ovulatory cows (**Figure 6.3**).

DISCUSSION

Follicle fate was not significantly associated with the presence of bacteriological contamination, pathogenic or potentially pathogenic bacteria or individual bacterial isolates. The decreasing proportion of cows with positive uterine bacterial culture over time is similar to previous reports (Sheldon and Dobson, 2004). The proportion of cows with known pathogenic and potentially pathogenic bacteria isolated from the uterus in at least one of the three uterine samples was slightly higher than previously reported (Williams et al., 2005) but the sampling schedule was different between the present study and the referenced study. The presence of bacteria was associated with follicular growth. Most bacterial isolates were associated with decreased follicle growth rate is in agreement with a previous report (Williams et al., 2007). *Escherichia coli* is a known uterine pathogen (Bicalho et al., 2010) and it is not surprising that isolation of this organism in the first uterine sample was associated with slower follicular growth. When individual *Escherichia coli* pathotype was tested, the individual pathotypes were not significantly associated with follicular growth. In the second uterine sample, the association between pathotype B1 and follicular growth

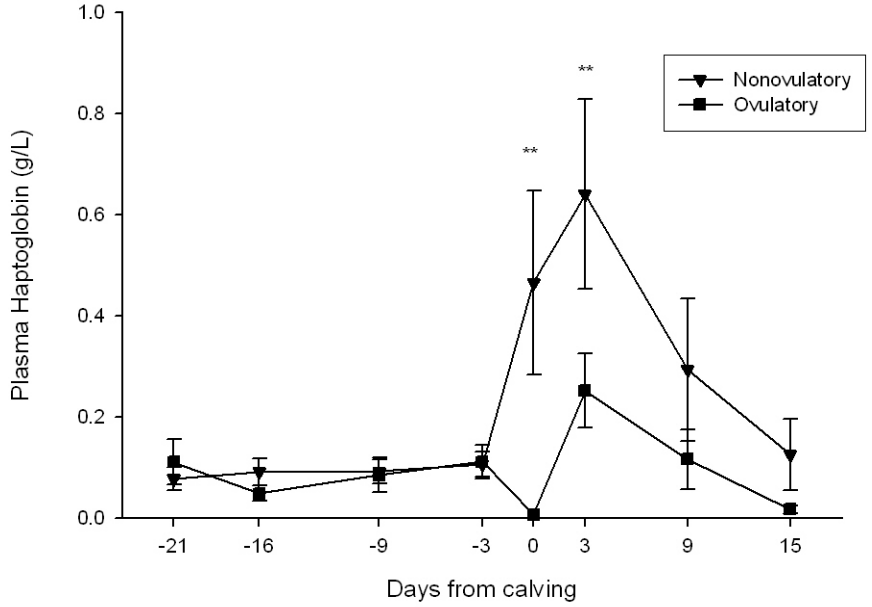

Figure 6.1. Plasma haptoglobin for ovulatory and non-ovulatory cows. There was a significant interaction between time and group with post-hoc test showing non-ovulatory cows having significantly higher levels at the day of calving and at 3 DIM (**) after Bonferroni correction.

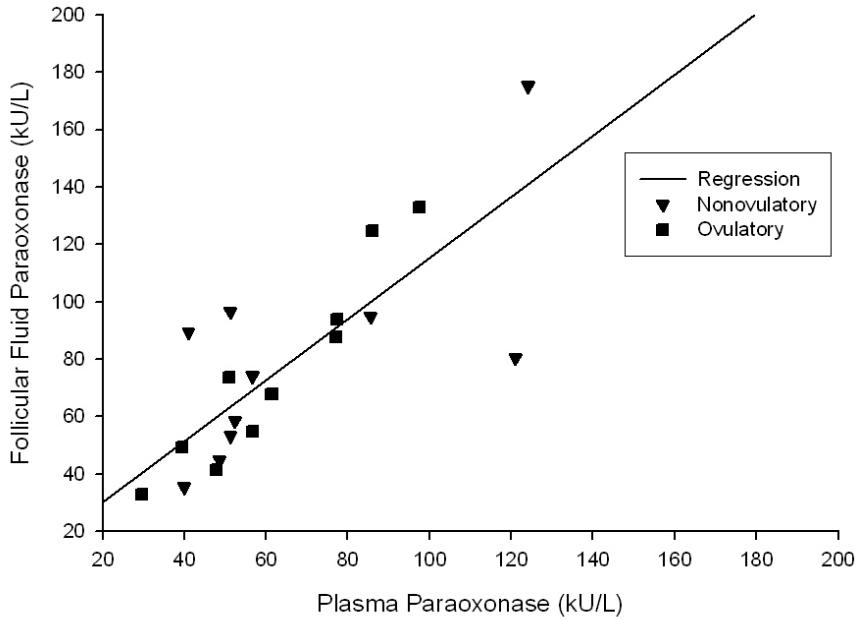

Figure 6.2. Scatterplot for follicular fluid and plasma paraoxonase. The association was linear and significant (1.06 ± 0.20; $P < 0.001$).

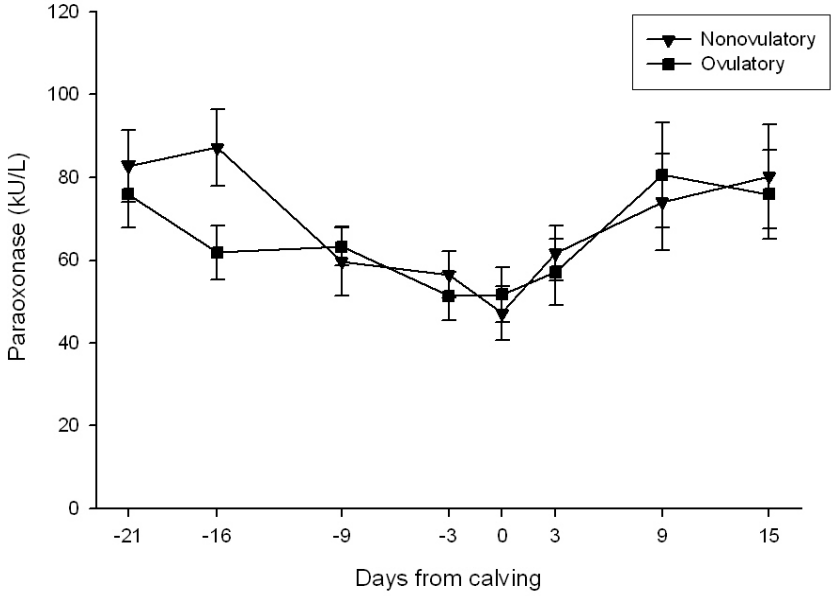

Figure 6.3. Plasma paraoxonase for ovulatory and non-ovulatory cows. There was no significant interaction between group and time and there were no significant difference in plasma paraoxonase levels between ovulatory and non-ovulatory cows.

was significant. However, cows with this pathotype had increased follicular growth rate. The B1 *Escherichia coli* pathotype has been associated with early postpartum uterine samples and uterine disease (Sheldon et al., 2010) and an explanation for this observation is not obvious. The other two bacterial isolates that were associated with reduced follicular growth rates were *Pasteurella spp.* and *Streptococcus uberis*. The classification for *Pasteurella spp*. includes *Pasteurella multocida*, a potential uterine pathogen and *Streptococcus uberis,* a non-hemolytic streptococcus which is also a potential uterine pathogen (Sheldon et al., 2002). Mycoplasma has been associated with reduced reproductive performance (Uhaa et al., 1990) in some studies but not in others (Petit et al., 2008). The presence of mycoplasma was only associated with reduced follicular growth in the second uterine sample.

There was a tendency for cows that had female calves to be ovulatory and cows that had acute puerperal metritis to be non-ovulatory. Calf weight was not associated follicle fate and the effect maybe due to factors other than the difference in size between male and female calves. The association between uterine neutrophil proportions on the day of calving and follicle fate was surprisingly stronger than acute puerperal metritis which is a severe uterine infection that results in systemic signs. High uterine pH on the day of calving was also associated with non-ovulatory status. In a previous study, high uterine pH was shown to be associated with uterine inflammation (Cheong et al., 2012). Uterine cytology and reagent strip results for the second and third uterine samples were not associated with follicle fate.

Endotoxin is able to be absorbed through the uterus into circulation (Peter et al., 1990a) then pass through follicle walls and have been detected in follicular fluid of cows with endometritis (Herath et al., 2007). Endotoxin affects steroidogenesis by impairing aromatization of androgens to estradiol but do not affect androgen production (Herath et al., 2007). In non-ovulatory cows, the follicular fluid

concentrations of estradiol were low but the concentrations of androsteinedione were also low (data from Chapter 5). There may be aromatase inhibition in non-ovulatory cows. However, the effects of endotoxin on follicle fate may be primarily through gonadotropin inhibition with non-ovulatory cows showing fewer LH pulses compared with ovulatory cows (Data in Chapter 5). Endotoxin administration has been shown to impair GnRH and LH release (Battaglia et al., 1997).

There was a significant difference in plasma haptoglobin levels between ovulatory and non-ovulatory cows on the day of calving and at 3 DIM. This pattern is similar to the observed patterns in cows with uterine disease (Huzzey et al., 2009). Cows with high uterine pathogen load have been shown to develop a rise in haptoglobin early postpartum (Williams et al., 2007). The difference in haptoglobin signifies a systemic acute phase response that occurs in non-ovulatory cows which is likely due to uterine inflammation.

Follicular fluid paraoxonase was significantly associated with plasma paraoxonase. Paraoxonase production is negatively correlated with haptoglobin and positive acute phase proteins (Bionaz et al., 2007) however, no difference was observed between ovulatory and non-ovulatory groups in the present study.

CONCLUSIONS

Uterine bacteria were not significantly associated with follicle fate although there were associations with follicle growth rates. Higher proportions of neutrophils and higher pH in the uterine samples at the day of calving were associated with non-ovulatory. Cows that had bull calves, and acute puerperal metritis tended to be non-ovulatory. Follicular fluid concentrations of endotoxin were higher in non-ovulatory cows which also had higher haptoglobin levels on the day of calving and at 3 DIM. Paraoxonase levels in follicular fluid were associated with circulating levels but were

not different between ovulatory and non-ovulatory cows.

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CHAPTER 7

GENERAL CONCLUSIONS

New knowledge generated from dissertation work

Chapter 2 describes the largest study to date in terms of the number of farms sampled for subclinical endometritis to determine prevalence of subclinical endometritis, identify cow- and herd-level risk factors, and determine reproductive outcomes. The cow-level risk factors identified were clinical ketosis, milk production and acute puerperal metritis. This was surprising because ketosis and milk production are related to energy balance and the association with subclinical endometritis was stronger than acute puerperal metritis which is a severe uterine infection. Subclinical endometritis is now viewed as a condition associated with negative energy balance. We also reported for the first time herd-level risk factors for subclinical endometritis which were: using straw as the bedding material in calving pens, and moving cows into free-stalls after calving (both being protective strategies associated with reduced within-herd prevalence of subclinical endometritis). The observed difference in reproductive consequence for primiparous cows and multiparous cows with subclinical endometritis was novel. Primiparous cows affected with this condition have similar reproductive performance to unaffected primiparous cows, while reproduction in multiparous cows is severely impaired by the condition. This finding suggests that enrolling primiparous cows for uterine sampling is not valuable as the information gained does not correlate with reproductive outcomes.

In the study reported in Chapter 3, the reagent strip test Multistix 10 SG was tested for the association with subclinical endometritis and reproductive outcomes. The reagent strip tests were found to be strongly associated with subclinical endometritis. However, when evaluating the performance of the reagent strip test as a

diagnostic test, the outcome was uninspiring for lack of sensitivity and specificity. Leukocyte esterase and pH reagent strip test were associated both with subclinical endometritis and impaired reproductive outcomes. Using a combination of leukocyte esterase and pH results, it was possible to classify a group of cows that were likely to have impaired reproductive performance and a group of cows that were likely to have good reproductive performance. However, there were a group of cows that were not able to be classified. This was the first report of uterine pH association with subclinical endometritis and association between leukocyte esterase and reproductive performance.

The study reported in Chapter 4 examined, for the first time, the possible effect of diagnostic low-volume uterine lavage on economically important parameters of reproductive performance, culling and milk production. There was no effect of sampling in multiparous cows. However; there was a tendency for sampled primiparous cows to have impaired reproductive outcomes. This finding, combined with the previous finding that the information gained from sampling primiparous cows were not associated with reproductive outcome, further strengthen the reasoning to avoid enrolling primiparous cows to studies on subclinical endometritis. This information is also useful for reassuring future participants in research on endometritis to allow access to their animals because this procedure is benign especially for multiparous cows.

In Chapter 5, a novel follicle fate prediction method was used to identify cows that will likely go on to ovulate or not to allow for sample collection. This was vital for the ability to study the mechanisms that result in the failure of these follicles to produce estradiol. In this study, follicular fluid was collected and analyzed for steroid hormone levels. It was not surprising that the follicular fluid concentrations of estradiol were lower in cows that do not show a rise in circulatory estradiol. The

novel finding was the lower follicular fluid androsteinedione concentrations in non-ovulatory cows. Estradiol is produced by aromatization of androsteinedione which is produced by the theca cells under the influence of LH. Lower LH pulse frequency has been implicated in delayed ovulation postpartum which was also observed in this study and the impaired androsteinedione production supports the role of LH in non-ovulatory cows. Postpartum glucose tolerance test found non-ovulatory cows to have slower glucose clearance and lower insulin response to glucose challenge compared with ovulatory cows whereas prepartum glucose tolerance test response were similar between ovulatory and non-ovulatory cows. Energy balance modeling showed the primary difference between nonovulatorty cows and ovulatory cows was the decrease in feed intake as ovulatory cows produced slightly more milk postpartum with the higher energy intake which caused the postpartum difference in energy balance between non-ovulatory and ovulatory cows to be smaller. The use of the Cornell Net Carbohydrate and Protein System also allowed simultaneous protein balance simulation. Protein balance was also significantly lower in non-ovulatory cows compared with ovulatory cows.

In Chapter 6, the association between uterine bacteria and the follicular function of the first dominant follicle postpartum was evaluated. No individual bacterial isolates or bacteria grouped as known uterine pathogens, potential uterine pathogens and non-uterine pathogens were associated with follicle fate. Several bacterial isolates were associated with reduced follicular growth rate, including: *Escherichia coli*, *Pasteurella spp*, *Streptococcus uberis*, and *Mycoplasma spp*. Uterine cytology samples on the day of calving had higher proportions of neutrophils and higher uterine pH in non-ovulatory cows compared with ovulatory cows. Follicular fluid endotoxin was higher in non-ovulatory cows. Circulating haptoglobin was significantly higher on the day of calving and at 3 days postpartum in non-

ovulatory cows compared with ovulatory cows. Taken together, uterine health and systemic inflammatory response is associated with impaired follicle function.

Future directions

The major risk factors associated with subclinical endometritis were energy balance related and to date no efficacious therapeutic strategies have been identified to treat affected animals. The future direction of subclinical endometritis research may be better management of the transition period of dairy cows to reduce future subclinical endometritis prevalence. Prevalence of subclinical endometritis in individual herds might be useful as an indicator of effective transition cow management. Supplementation of minerals, choline and conjugated linoleic acid, and other potential strategies may be fruitful as would novel sampling strategies to simplify diagnosis of affected cows.

Perhaps the most important finding in the dissertation work is the validation of follicle fate prediction for the first dominant follicle postpartum. This allows ovarian follicle samples to be collected and analyzed to elucidate dysfunction in non-ovulatory follicles. One area to pursue is the identification of follicular factors in the steroidogenic dysfunction. Culturing follicle wall sections from ovulatory and non-ovulatory follicles, will allow functional testing of follicular processes by manipulating culture conditions, for example to determine if the follicular cells are able to respond equally to gonadotropin stimulation. It will also be possible to determine the difference in gene transcription in ovulatory and non-ovulatory cells and using bioinformatic tools will be useful to determine pathways that are disrupted in these cells.

The association between uterine health and follicular function should be further investigated. The concentrations of endotoxin in follicular fluid are much

higher than in circulation and the reason for this is unclear. Tumor necrosis factor α is an important cytokine that affects a variety of cellular functions including insulin action, steroidogenesis, apoptosis and the acute phase response that are associated with follicle function and its role should be further characterized.

The genetics of early resumption of ovarian cyclicity postpartum is another area to pursue. The heritability of reproductive traits is generally low in dairy cows and is part of the reason why reproductive performance has not been traditionally selected for by bull studs. However, the heritability of physiological outcomes such as early resumption of cyclicity is relatively higher and suggests some genetic component to the condition. The introduction of the high density single-nucleotide polymorphism chip allows a higher resolution and ability to detect quantitative trait loci associated with early resumption of ovarian cyclicty with fewer cows. The identification of associated genes not only will be useful as a potential selection tool but also to uncover the pathophysiology of early postpartum anovulation.

General conclusions

Inflammation is present in the uterus of all cows after calving and this is part of normal uterine involution. Upon completion of uterine involution, the uterine inflammation is cleared and the uterus is ready to support the next pregnancy. Many cows fail to complete this physiological process efficiently and have delayed clearance of uterine inflammation. The primary factors associated with delayed clearance of uterine inflammation were: energy related (ketosis and milk production) and uterine health related (metritis). Uterine inflammation is diagnosed by cytological determination of high proportion of neutrophils, and thus leukocyte esterase, in uterine samples but also by elevated pH. This condition is subclinical endometritis and multiparous cows with subclinical endometritis have impaired reproductive

performance. Herd factors of moving cows directly into freestalls and the use of straw as the calving pen bedding material is associated with reduced prevalence of subclinical endometritis in the herd and thus associated with increasing the ability of cows to clear uterine inflammation. The diagram in figure 7.1 summarizes the factors associated with delayed clearance of uterine inflammation.

Most cows that have delayed resumption of ovarian cyclicity develop an ovulatory size follicle shortly after calving but the follicle fails to produce a rise in circulating estradiol. The LH pulse frequency and lower follicular androsteinedione levels indicate an impairment of theca cell function. Non-ovulatory cows also have insulin resistance. Insulin is required for the ability of theca and granulosa cells to respond to gonadotropin stimulation. Non-ovulatory cows also have lower energy balance which has been shown to be associated with decreasing the gonadotropin release by the hypothalamic-pituitary axis which was seen in non-ovulatory cows.

Uterine health conditions also affect the resumption of ovarian cyclicity where the presence of higher polymorphonuclear leukocyte and higher pH in uterine samples collected at the day of calving is associated with non-ovulation of the first dominant follicle postpartum. Bacterial endotoxin was found in follicular fluid of non-ovulatory cows and has been shown to impair the ability of granulosa cells to aromatize androgens to estradiol and lower follicular fluid estradiol was found suggesting a potential direct impairment of granulosa cell function. Endotoxin has been shown to impair the hypothalamic-pituitary axis directly but cows that have endotoxin also have lower appetite which was seen in non-ovulatory cows which indirectly impairs the hypothalamic-pituitary axis through lowering energy balance. In addition, endotoxin is a potent stimulator of the inflammatory system inducing, among others, TNFα release. Non-ovulatory cows have systemic inflammation as shown by the elevated plasma haptoglobin levels. The ability of TNFα to impair insulin function may

contribute to the insulin resistance seen in non-ovulatory cows. The diagrammatic summary for factors associated with delayed ovarian cyclicity is shown in figure 7.2.

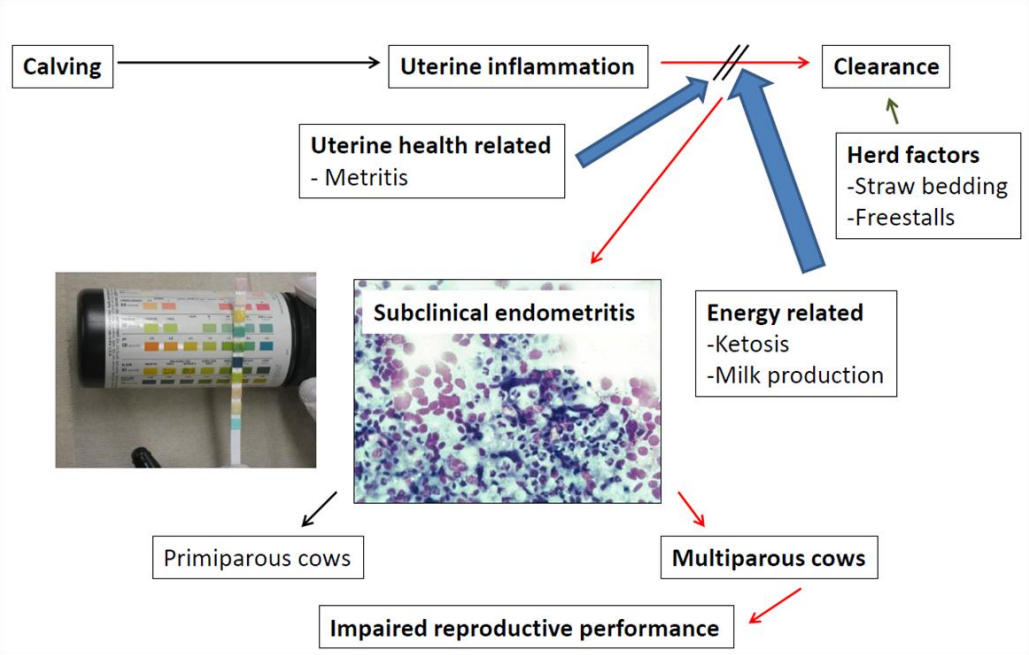

Figure 7. 1 Diagrammatic summary for the factor associated with delayed clearance of uterine inflammation

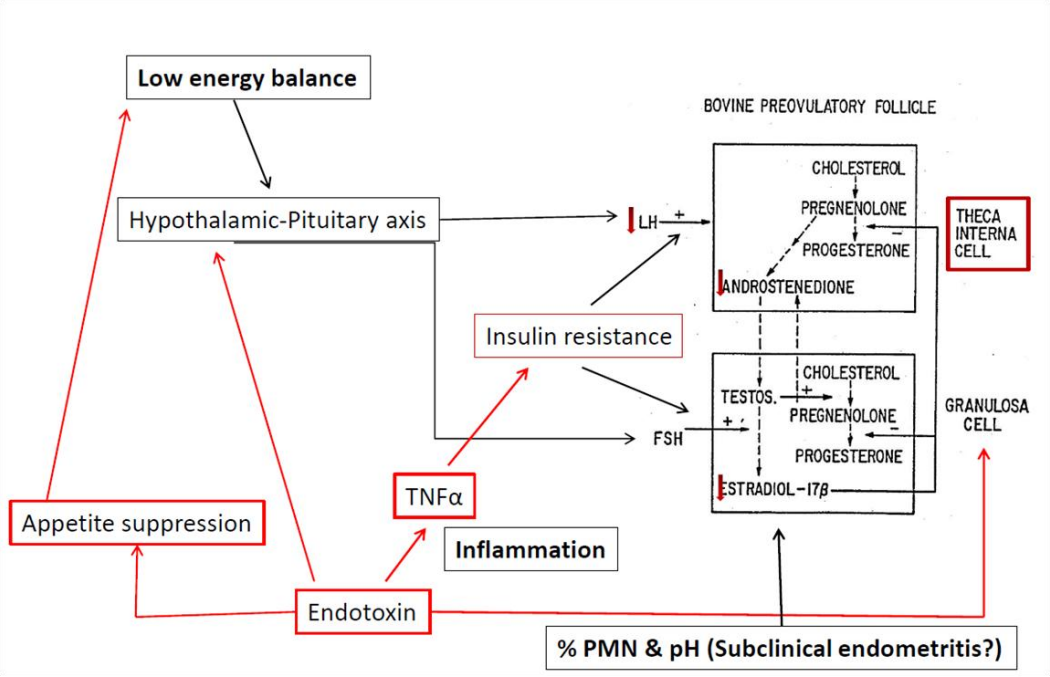

Figure 7. 2. Diagrammatic summary for the factors associated with delayed resumption of ovarian cyclicity